

CATALYTIC PROTON TRANSFER IN THE LIVER-ALCOHOL-DEHYDROGENASE REACTION

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INTRODUCTION

Living organisms convert energy and matter for various life-maintaining purposes and must be succesful in carrying out a large variety of chemical reactions. These reactions generally proceed very fast in spite of the "mild" physiological conditions prevailing. They are integrated into different metabolic and physiological processes and subjected to various forms of regulation. These phenomena are intimately linked to the catalytic activity of a specific group of proteins, called enzymes. Enzymes participate in virtually all cellular processes and an understanding of the biochemistry of such processes requires knowledge of the enzyme systems involved.

The biosynthesis of enzymes is subjected to direct genetic control and forms an essential part of the heredity pattern of all organisms. Millions of years of evolutionary refinement have endowed enzymes with outstanding catalytic properties. Each of them is designed to fulfil a catalytic function of metabolic importance. In so doing they are by far more efficient than the organic or inorganic catalysts generally employed in laboratorial and industrial chemical synthesis. Investigations of the structure and function of various enzymes are not only of general biochemical interest, but may also provide valuable insights into the entire field of chemical reactivity.

The present thesis concerns the zinc-containing enzyme Alcohol Dehydrogenase from horse liver (abbreviated LADH). This classical enzyme was first crystallized by Bonnichsen and Wassén in 1948 and shortly afterwards subjected to pioneering enzymological studies by Theorell and his coworkers. Since then, it has been the subject of extensive studies which have put LADH among the most wellstudied enzymes. An abundance of kinetic, physico-chemical and structural information about LADH is now available. The actual state of knowledge in this field has recently been reviewed (1,2).

Alcohol dehydrogenases are of particular enzymological interest as representatives of the large group of enzymes which catalyze redox reactions between central metabolites and the nicotinamide adenine dinucleotide coenzymes. Alcohol dehydrogenase from horse liver acts upon a variety of primary and secondary alcohols which are oxidized by NAD^+ into the corresponding aldehydes and ketones according to the stoichiometric relationship



The enzyme is also efficient in catalyzing the reverse reaction of carbonyl group reduction by NADH.

As indicated by Eqn(1), LADH mediates the net removal of two hydrogen atoms from an alcohol substrate by a process of combined hydride ion and proton transfer. The hydride ion is transferred to the nicotinamide ring of the coenzyme and the proton is released ultimately to the reaction medium. Crystallographic and solution studies have clarified essential features of the corresponding reaction mechanisms. Hydride transfer is known to occur directly between the enzyme-bound substrate and coenzyme which require formation of a ternary enzyme-coenzyme-substrate complex during catalysis. The static aspects of such complex formation have been elucidated in considerable detail. The dynamics of complex formation and catalytic dehydrogenation, however, are less well characterized. In particular the detailed mechanism of the proton transfer process and the functional role of the enzyme-bound zinc ions have been under vigorous debate. The present thesis deals mainly with the latter two aspects and summarizes the results published in the papers listed on the opposite page.

- I. Kinetic Transients in the Reduction of Aldehydes
Catalyzed by Liver Alcohol Dehydrogenase
Jan Kvassman and Gösta Pettersson (1976)
Eur. J. Biochem. 69, 279-287.
- II. Effect of pH on Coenzyme Binding to Liver Alcohol
Dehydrogenase
Jan Kvassman and Gösta Pettersson (1979)
Eur. J. Biochem. 100, 115-123.
- III. Effect of pH on the Process of Ternary-Complex
Interconversion in the Liver-Alcohol-Dehydrogenase
Reaction
Jan Kvassman and Gösta Pettersson (1978)
Eur. J. Biochem. 87, 417-427.
- IV. Effect of pH on the binding of Decanoate and
Trifluoroethanol to Liver Alcohol Dehydrogenase
Jan Kvassman and Gösta Pettersson (1980)
Eur. J. Biochem. 103, 557-564.
- V. Unified Mechanism for Proton-Transfer Reactions
Affecting the Catalytic Activity of Liver Alcohol
Dehydrogenase
Jan Kvassman and Gösta Pettersson (1980)
Eur. J. Biochem. 103, 565-575.
- VI. Substituent Effects on the Ionization Step
Regulating Desorption and Catalytic Oxidation of
Alcohols Bound to Liver Alcohol Dehydrogenase
Jan Kvassman, Anita Larsson and Gösta Pettersson
(1981) Eur. J. Biochem. 114, 555-563.

INTERMEDIATES IN THE CATALYTIC REACTION

LADH is a dimeric enzyme with structurally identical subunits. Each subunit contains two tightly bound zinc ions, one of which is known to be catalytically essential (1). The sequence of the 374 amino acid residues of the LADH subunit was determined by Jörnvall (3). Brändén and coworkers have applied x-ray crystallography for structural determination of the free LADH molecule and a number of its binary and ternary complexes (4,5). In the latter structural investigations it was concluded that each subunit of the LADH molecule is divided into two domains separated by a deep cleft which accommodates the nicotinamide moiety of the coenzyme. The catalytically essential zinc ion is bound by three protein ligands at the bottom of this cleft and coordinates a water molecule as a fourth inner-sphere ligand. This water molecule projects into a hydrophobic region which is accessible to substrates from solution through a channel lined by non-polar sidechains. In each subunit, the outer region of this channel is lined also by residues from the other subunit. Some of these aspects of the structure of LADH is schematically depicted in Fig.1

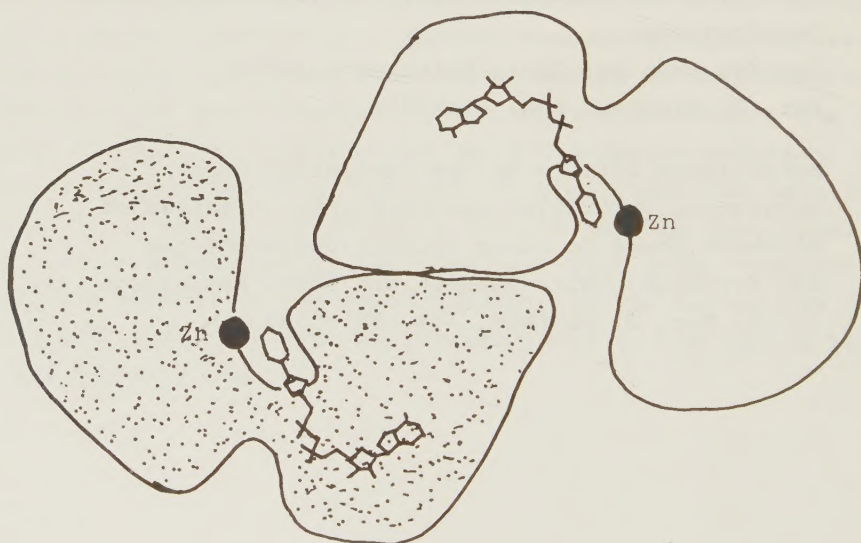
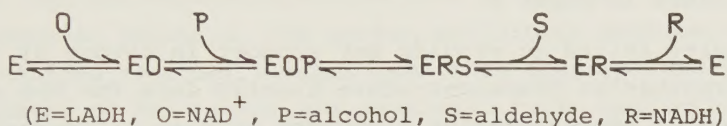


Fig.1. Schematic diagram of a section through the LADH molecule with projections of bound coenzyme.

The crystallographic investigations establish that the structural prerequisites are at hand for LADH-catalysis by a ternary-complex mechanism involving direct hydride transfer between substrate and coenzyme. They lend strong support to the idea that alcohol and aldehyde substrates are coordinated by the active-site zinc ion in the productive ternary complexes formed during catalysis. As will be discussed in more detail below, this means that an understanding of the catalytic reaction mechanism requires characterization of the properties of zinc-bound water and zinc-bound substrates.

The "Half-sites" Reactivity Hypothesis (Paper I).

Steady-state kinetic studies of LADH (6) have established that the enzyme operates by an effectively ordered ternary complex mechanism according to the following reaction scheme:



Scheme 1

Crystallographic data do not exclude ligand-induced subunit interactions during LADH-catalysis, but the above mentioned kinetic studies, as well as equilibrium and stop-flow kinetic studies of coenzyme binding (7-10), have failed to provide evidence for any non-equivalence of the two subunits. Likewise, transient-state kinetic data for enzymic ethanol oxidation and acetaldehyde reduction are consistent with catalysis at equivalent sites (11).

In the early 1970th, however, a series of investigations by Bernhard *et al.* (12-14) reported that the enzymic reduction of various aromatic aldehydes and aldehyde analogues is characterized by two exponential transients of about equal amplitudes. The interpretation of this observation was that LADH exhibits a "half-sites" reactivity. The initial rapid transient was envisaged to reflect hydride transfer at one subunit only. Hydride transfer at the second subunit was assumed to be slow and to be triggered by alcohol desorption from the first reacting subunit.

The half-sites reactivity hypothesis was criticized by several groups(15-17). In particular, a theoretical analysis was presented by Pettersson(17) which showed that reported transient-state kinetic data for the LADH-system might be entirely consistent with enzyme catalysis by an ordered mechanism involving equal and independent sites (Scheme 1). According to this analysis, biphasic transients will appear in aldehyde reduction by LADH when there is a significant accumulation of the ternary enzyme-NAD⁺-alcohol complex during the presteady-state phase of the reaction; i.e. when the alcohol products dissociate slowly relatively to the rate of their formation.

The theory described by Pettersson provided relationships for the concentration dependence of rates and amplitudes of the transients governing the enzymic reaction. This made it possible to test experimentally the validity of Scheme 1. Such tests are reported in Paper I.

The results failed to provide any support in favour of the half-sites hypothesis. Transient-state kinetic data for the enzymic reduction of representative aldehydes were in all respects tested found to adhere to the predictions of Scheme 1. The transient-state kinetics of enzymic alcohol oxidation (reported in Paper III) were similarly shown to be fully consistent with Scheme I.

Subsequent kinetic studies of the catalytic reaction mechanism, therefore, were performed and evaluated with the assumption that Scheme 1 applies.

PROTON TRANSFER STEPS IN THE LADH REACTION

As prescribed by Eqn(1) one proton is released per alcohol molecule oxidized in the LADH reaction. Although the obvious ultimate origin of this proton is the alcohol hydroxyl group, the proton may initially be accepted by a basic group at active-site and proton transfer to solution occur at any stage of the catalytic reaction sequence. In principle, it should be easy to decide what step in Scheme 1 is coupled to catalytic proton release; this step must exhibit a corresponding direct dependence on the hydrogen ion concentration. The practical situation, however, is complicated by the fact that several proton dissociation equilibria are known to affect the catalytic activity of LADH(1). A detailed understanding of the catalytic dehydrogenation mechanism, therefore, requires a characterization of the number and nature of protolytic groups involved and of the effects of the corresponding ionizations on the reactivity of intermediates in Scheme 1. The proton equilibria must be combined into a unified reaction scheme, such that they account for the net transfer of one proton per catalytic cycle and active-site.

To arrive at such an understanding, we carried out a series of investigations (Paper II-VI) of the effects of pH on individual reaction steps in Scheme 1. These investigations have emerged into a mechanistic proposal which gives a unified account for the stoichiometry and pH-dependence of the LADH-reaction over the pH-range 6-10.

Effects of pH on Coenzyme Binding (Paper II).

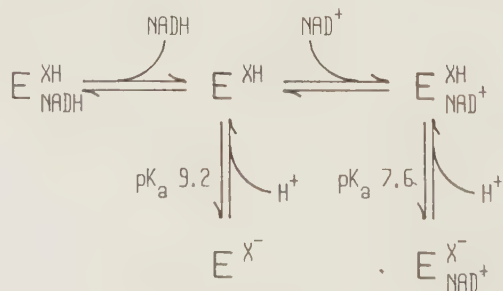
The catalytic activities of NAD-dependent dehydrogenases have in common the combined hydride ion and proton transfer reactions. Considering the ionic/zwitterionic character of the NADH/NAD^+ redox couple, ionization of protein groups in the vicinity of the coenzyme binding sites would in general be expected to affect the stability of the enzyme-coenzyme complexes. As concerns LADH-catalysis, attention has been drawn to the possible role of such ionizations in catalytic proton transfer. This derives from the fact that LADH, in contrast to other wellstudied dehydrogenases, exhibits a pH-dependent affinity ratio for NADH and NAD^+ , such that displacement of bound NAD^+ by NADH is coupled to proton withdrawal from solution (1,6).

Stop-flow kinetic studies, however, have led to contradicting conclusions about the ionization steps which regulate coenzyme association to LADH. DeTraglia *et al.* (18) reported that association of both NADH and NAD^+ requires protonation of a pK_a -9.5-group in the free enzyme. Shore *et al.* (19), on the other hand, reported that these steps are dependent on different ionizations corresponding to pK_a -values of 9 and 10 for the association of NADH and NAD^+ respectively.

Another controversy concerns the effect of pH on NAD^+ desorption from the enzyme- NAD^+ complex. Shore *et al.* (20) found that a pK_a -7.6-group affects the stability of the enzyme- NAD^+ complex as well as the association of substrate analogues to this complex. They proposed that NAD^+ desorption requires protonation of the pK_a -7.6-group. Direct determination of the effect of pH on the off-velocity constant for NAD^+ binding, however, led DeTraglia *et al.* (18) to conclude that dissociation of NAD^+ requires protonation of a less acidic group.

These ambiguities obviously interfere with the attempt to deduce the mechanism of catalytic proton transfer in the LADH reaction, and the investigation reported in Paper II was performed in order to resolve them. In Paper II we carried out a theoretical analysis of the transient-state kinetics of ligand-displacement reactions. Methods based on this analysis were applied to obtain reliable estimates of on- and off-velocity constants for coenzyme binding to LADH over the pH-range 6-10. The kinetic results were checked by titrimetric methods.

Data reported in Paper II provide evidence that all major effects of pH on coenzyme binding to LADH over the investigated pH-range are accounted for by two proton dissociation equilibria as shown in Scheme 2



Scheme 2

According to Scheme 2, association of both coenzyme forms requires protonation of a pK_a -9.2-group in the free enzyme. Desorption of NADH is essentially independent of ionizations over the investigated pH-range, while NAD^+ desorption requires protonation of a pK_a -7.6-group in the enzyme- NAD^+ complex, as suggested by Shore *et al.*

The kinetic results summarized in Scheme 2 provided no direct information about the nature of the ionization steps which affect coenzyme-binding to LADH. The idea was early put forward, however, that effects of pH on NAD^+ binding are attributable to ionization of a single enzymic group, tentatively identified as a water molecule bound to the active-site zinc ion (21). Crystallographic and solution studies have provided strong evidence in favour of this idea (1,22). As argued in Paper II, there seems to be little doubt that the pH-dependence of NAD^+ dissociation derives from ionization of zinc-bound water with a pK_a -value perturbed to 7.6 through electrostatic interaction with NAD^+ . Insufficient information was available at that time to draw any firm conclusion as to the actual pK_a of zinc-bound water in the free enzyme (*i.e.* as to the identity of the pK_a -9.2-group which regulates coenzyme association). Recent investigations (23,24), however, have provided strong evidence for the identity of the pK_a 9.2 and 7.6 groups. The following presentation, therefore, will be based on the assumption that both ionization steps in Scheme 2 reflect ionization of zinc-bound water.

Effects of pH on Ternary-Complex Interconversion (Paper III).

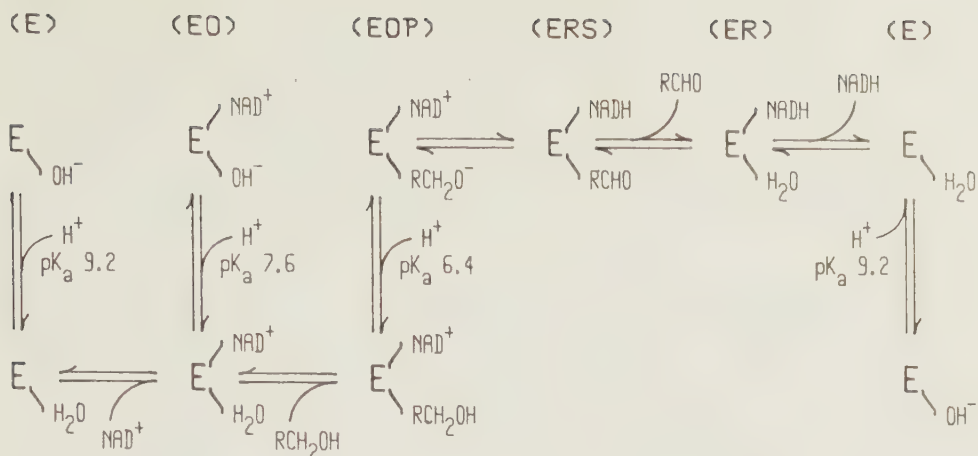
Transient-state kinetic studies of alcohol oxidation by LADH have led to different conclusions about the effects of pH on the hydride transfer step in this reaction. Brooks *et al.* (25), reported that hydride transfer from ethanol to NAD^+ depends on ionization of an enzyme functional group exhibiting a pK_a of 6.4. McFarland *et al.* (26), on the other hand, could not detect any significant effect of pH on hydride transfer from benzyl alcohol to NAD^+ or from NADH to aromatic aldehydes over the pH-range 6-10. The latter results were taken to exclude base catalysis of alcoholoxidation as well as acid catalysis of aldehyde reduction, by any group with a pK_a between 6 and 10.

The investigation reported in Paper III was carried out in order to obtain more definite knowledge about the effects of pH on the ternary-complex isomerization step in LADH-catalysis. Benzyl alcohol oxidation was examined by stop-flow techniques between pH 5 and 9, and benzaldehyde reduction was similarly examined over the alkaline pH-range. The transient-state kinetic parameters determined were evaluated by application of the relationships derived by Pettersson (17).

The results relating to the enzymic process of benzaldehyde reduction support the conclusion of McFarland *et al.* that there is no pronounced effect of pH on hydride transfer from NADH to aldehydes.

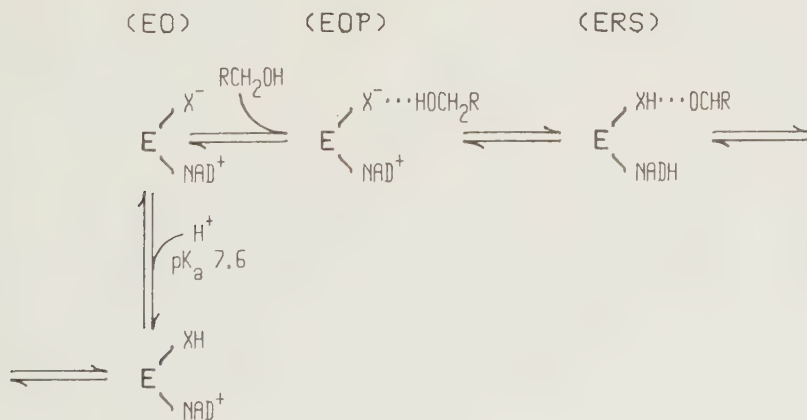
As concerns the reverse process of enzymic benzyl alcohol oxidation, our data agree well with those reported by McFarland *et al.* over the more limited pH-range examined by these authors. Below pH 6, however, we observed a marked decrease in the pre-steady-state rate of benzyl alcohol oxidation. This effect of pH was shown to be attributable to a single ionization which affects hydride transfer from the alcohol to NAD^+ and exhibits a pK_a -value of 6.4. The analogy to the situation in enzymic ethanol oxidation (25) suggests that the pK_a -6.4-effects have a common mechanistic origin.

The observed assymetric pH-dependence of ternary-complex isomerization in LADH-catalysis implies that the enzyme- NAD^+ -alcohol complex, but not the enzyme-NADH-aldehyde complex, participates in an obligatory proton transfer step. This suggested to us that the catalytic step of proton transfer may take place at the ternary-complex level, being uncoupled from the actual step of hydride transfer and involving direct proton transfer from the enzyme-bound alcohol. Considering the evidence indicating that enzyme-bound substrates are directly ligated to the active-site zinc ion (27-29), we proposed that the pK_a -6.4-ionization reflects an alcohol/alcoholate ion equilibration of the zinc-bound substrate. The results in Paper II and III may then be combined into the following minimal scheme for LADH-catalysis:



Scheme 3

Previously published mechanistic proposals (20,1,30) invariably have attributed a crucial role in catalytic proton transfer to the ionizing group which affects coenzyme binding (*i.e.* to zinc-bound water). The NAD^+ -induced perturbation of the pK_a of this group results in proton liberation at binary complex level, and proton release due to NAD^+ binding has been assumed to represent the obligatory step of catalytic proton transfer during alcohol oxidation. In other words, alcohol binding to the enzyme- NAD^+ complex has been envisaged to involve the ionized form of this complex, as indicated in Scheme 4. This scheme was proposed by Shore *et al.* (20) and, with some minor modifications (1,30), has been widely accepted.



Scheme 4

According to our proposal in Scheme 3, however, ionizations affecting coenzyme binding to LADH are side reactions and do not contribute to the net transfer of protons in the catalytic cycle. Equilibration of the enzyme-NAD⁺-alcohol complex represents the obligatory proton transfer step. The assumption that this step involves deprotonation of the alcohol substrate is mechanistically sound, since this would facilitate hydride transfer to NAD⁺. Moreover, it may be noted that hydride transfer from NADH to an aldehyde substrate results in the corresponding alcoholate ion and NAD⁺. Electrostatic attraction between the latter two species provides a likely explanation why alcohol dissociation requires protonation of the pK_a-6.4-group. These and other implications of the mechanism in Scheme 3 will be further discussed in the following sections.

Effects of pH on Ligand Binding to the Enzyme-NAD⁺ Complex (Paper IV).

The experimental support for Scheme 4, reported by Shore *et al.* (20), was obtained by studying the effects of pH on the binding of the inhibitors trifluoroethanol and decanoate to the enzyme-NAD⁺ complex. The results demonstrate that ternary-complex formation with trifluoroethanol(decanoate) is thermodynamically favoured(disfavoured) by ionization of the pK_a-7.6-group. These findings were taken to indicate preferential binding of trifluoroethanol(decanoate) to the enzyme-NAD⁺ complex form where the pK_a-7.6-group is deprotonated(protonated).

The interpretation presented by Shore *et al.*, is based on the implicit assumption that the observed pH-dependence of the equilibrium binding constants reflects exclusively the reactivity of the binary enzyme-NAD⁺ complex (*i.e.* pH-effects on the on-velocity constants for ligand binding to this complex). The equilibrium binding constants, however, may reflect also effects of pH on the corresponding off-velocity constants. This means that the equilibrium data reported by Shore *et al.* could be given an alternative interpretation in view of our mechanistic proposal in Scheme 3; alcohols might bind to the protonated form of the enzyme-NAD⁺ complex and the resulting ternary complexes might be stabilized by subsequent deprotonation.

In Paper IV, therefore, we performed a complementary study of the effects of pH on trifluoroethanol and decanoate binding to the enzyme-NAD⁺ complex. The corresponding off-velocity constants were determined between pH 3 and 9 by ligand displacement methods analogous to those employed in Paper II. Pyrazole, a potent alcohol competitive inhibitor (31), was used as displacing reagent and reporter ligand.

These studies indicated that there is no significant effect of pH on the rate of decanoate dissociation from the ternary complex formed with this ligand, enzyme and NAD⁺. On the other hand, dissociation of trifluoroethanol from the corresponding ternary complex was found to be markedly dependent on the hydrogen ion concentration and to require protonation of a pK_a-4.3-group. Considering these observations, it follows from the equilibrium data reported by Shore *et al.* (20), that trifluoroethanol and decanoate both combine with the enzyme-NAD⁺ complex form where the pK_a-7.6-group is protonated. Ionization of the pK_a-4.3-group stabilizes the enzyme-NAD⁺-trifluoroethanol complex and superimposes on the destabilizing ionization of the pK_a-7.6-group. The two effects could not be distinguished by Shore *et al.* in the more limited pH-range investigated by these authors.

There is crystallographic evidence that trifluoroethanol and substrate alcohols form structurally analogous complexes with enzyme and NAD⁺ (32). It seems reasonable, therefore, to assume that the pK_a-4.3-ionization in the trifluoroethanol complex is mechanistically analogous to the pK_a-6.4-equilibration of the corresponding complexes with substrate alcohols (ethanol and benzyl alcohol). According to our interpretation of the latter ionization (Scheme 3), this would mean that bound trifluoroethanol participates in an alcohol/alcoholate ion equilibration. By analogy to the discussion of Scheme 3, this explains why dissociation of trifluoroethanol requires protonation of the pK_a-4.3-group. The absence of pH-effects on decanoate dissociation is easily understood, since this ligand lacks a corresponding ionizing group.

A Unified Mechanism (Paper V).

Results described above provide evidence that effects of pH on coenzyme binding and ternary-complex interconversion during LADH-catalysis over the pH-range 6-10 are accounted for by three different proton transfer steps which are linked to the catalytic sequence of reactions as indicated by the mechanism in Scheme 3. They further demonstrate that pH-effects on the binding of inhibitory substrate analogues can be well understood in view of this scheme. The additional information required to establish that Scheme 3 represents a coherent mechanistic description of the proton transfer reactions affecting LADH-catalysis concerns the binding of alcohols and aldehydes which function as substrates for the enzyme. To obtain such information, we examined effects of the hydrogen ion-concentration between pH 6 and 10 on transient and steady-state kinetic parameters which are known to be more or less directly related to substrate binding in the enzymic reaction (Paper V).

The transient-state kinetic part of the investigation in Paper V provided information about the effect of pH on the step of alcohol dissociation in the enzymic reduction of β -naphthaldehyde and benzaldehyde. The results clearly demonstrate that dissociation of the corresponding alcohols exhibits a dependence on the hydrogen ion concentration which is analogous to that observed for trifluoroethanol. Dissociation of β -naphthyl alcohol requires protonation of a pK_a -6.6-group and dissociation of benzyl alcohol requires protonation of a group which must have a pK_a below 7.

Kinetic data available for the benzyl alcohol/benzaldehyde substrate system made possible an unambiguous interpretation of the pH-effects on steady-state rate parameters determined for these substrates. The information obtained by such measurements indicate that the protolytic group which regulates dissociation of benzyl alcohol have a pK_a of 6.4. The reverse process of ternary complex formation was shown to proceed via alcohol binding to the enzyme- NAD^+ complex form where the pK_a -7.6-group is protonated. It was also concluded from these measurements that no ionizations in the enzyme-NADH complex affect aldehyde binding over the investigated pH-range.

These results establish the descriptive validity of Scheme 3 with respect to the effects of pH on substrate binding during LADH-catalysis.

Stop-flow kinetic examinations of the LADH-reaction performed in low buffer solution with pH indicator added have shown that displacement of NAD^+ by NADH at pH 8.8 is accompanied by a net uptake of one proton per NAD^+ displaced (33). This is consistent with the mechanism in Scheme 3. They have failed, however, to provide evidence that proton transfer is kinetically coupled to ternary-complex interconversion at this pH. According to Scheme 3, however, proton transfer at the binary and ternary complex level can be kinetically distinguished by stop-flow techniques only under experimental conditions such that there is a significant accumulation of the protonated form of the enzyme- NAD^+ -alcohol complex in the presteady-state phase of the enzymic reaction.

Proton release/uptake measurements performed under such conditions are described in Paper IV and V. The results obtained at pH-values around 6 demonstrate that hydride transfer to(from) substrate in the LADH-reaction is kinetically coupled to an uptake(release) of protons in the amount predicted by Scheme 3. Moreover, they show that the displacement of trifluoroethanol by an alcohol substrate (and *vice versa*) at this pH is kinetically coupled to uptake(release) of the expected amount of protons. These observations lend crucial support to our mechanistic proposal in Scheme 3.

Our investigations, therefore, have established that Scheme 3 represents a coherent and unified description of proton transfer reactions affecting the catalytic efficiency of LADH over the pH-range 6-10.

Evidence for the Suggested Alcohol/Alcoholate ion Equilibration (Paper VI).

Results described in Paper II-V establish that the enzyme- NAD^+ -alcohol complex participates in an obligatory proton dissociation equilibrium which regulates both oxidation and desorption of the alcohol ligand. Determinations of the corresponding acid dissociation constants, however, have failed to provide evidence which automatically justifies the assumption in Scheme 3,

that this effect of pH derives from an alcohol/alcoholate ion equilibration of the enzyme-bound alcohol. The pK_a -values determined for the ionization regulating the reactivity of the productive enzyme- NAD^+ -alcohol complexes hitherto examined are all close to 6.4. This might be taken to favour the alternative possibility that the pH-effect derives from ionization of a substrate independent group on the enzyme. The pK_a -shift in the corresponding complex with trifluoroethanol might reflect a different mode of binding of this inhibitory alcohol rather than being attributable to a substituent effect on the proton dissociation step.

The results described in Paper VI, however, clearly demonstrate that the critical ionization of the enzyme- NAD^+ -alcohol complex involves deprotonation of the alcohol ligand. Two alcohols (2-chloroethanol and 2,2-dichloroethanol), with electronic properties intermediate between those of ethanol and trifluoroethanol, were examined with respect to the ionization properties of the corresponding enzyme- NAD^+ -alcohol complexes. Alcohol association, on formation of these complexes, was invariably found to require protonation of the pK_a -7.6-group while the ternary-complex proton equilibrium affecting alcohol desorption was found to be ligand dependent. Chloroethanol dissociates exclusively from the complex form where a pK_a -5.4-group is protonated while dissociation of dichloroethanol requires protonation of a pK_a -4.5-group. As predicted by Scheme 3, ionization of the pK_a -5.4-group was found to regulate the comparatively slow hydride transfer step in the enzymic oxidation of chloroethanol. The latter observation excludes the possibility that the pK_a -shift in the chloroethanol complex (and, by inference, those observed in the dichloro- and trifluoroethanol complexes) derives from an improductive mode of binding of the alcohol.

The pK_a -values now determined, and those previously found for the corresponding ionization in the ternary complexes formed with ethanol(=6.4) and trifluoroethanol(=4.3), were shown to be linearly related to the pK_a of the free alcohol ligands (34) with a regression coefficient (Brönstedt α -value) of about 0.6. This provides direct evidence that the effect of pH on the reactivity of the enzyme- NAD^+ -alcohol complexes reflects an alcohol/alcoholate ion equilibration of enzyme-bound alcohol.

MECHANISTIC IMPLICATIONS

The previous conception that catalytic proton exchange with solution is associated with coenzyme binding in the LADH-reaction would imply that the catalytic reaction mechanism of this enzyme is basically different from that of other dehydrogenases (1,6). Our investigations, however, have provided strong evidence that LADH, in analogy to other dehydrogenases, operates by a mechanism where proton transfer between substrate and solution is associated with ternary-complex isomerization. Proton liberation upon NAD^+ binding is a consequence of the fact that the active-site of LADH lacks a pre-existing negative charge which could counterbalance the positive charge of the coenzyme; the corresponding effect is achieved by a NAD^+ -induced ionization of zinc-bound water in the binary complex and of zinc-bound alcohol in the catalytically productive ternary complex. Since the latter ionization is on the main catalytic route, NAD^+ has the additional "coenzymic" effect of facilitating deprotonation of the alcohol substrate. The analogous ionization of zinc-bound water is an inevitable consequence of the fact that water and alcohols are chemically related. This ionization, however, represents a side reaction since alcohol association requires reprotonation of the zinc-bound hydroxide ion.

The idea has been advanced (30) that substrate binding to the active-site zinc ion in LADH involves a change in the coordination number of the metal from four to five. The inner-sphere water molecule has been assumed to remain zinc-bound on ternary complex formation and to act as an acid/base catalyst of hydride transfer to or from substrate. Our results argue strongly against this possibility. They indicate that base catalysis of hydride transfer in the enzyme- NAD^+ -alcohol complex (if being at hand) must be effected by a group with a pK_a (≤ 6.6) lower than that (7.6) of zinc-bound water in the binary enzyme- NAD^+ complex. A penta-coordinate water molecule, however, would be expected to exhibit a higher pK_a than a tetra-coordinate one. The presence in the enzyme- NAD^+ -alcohol complex of a penta-coordinate water, in fact, would seem to be catalytically disadvantageous. It would tend to increase the pK_a of a zinc-

bound alcohol substrate, thus disfavours the obligatory step of alcoholate ion formation which precedes hydride transfer during catalytic alcohol oxidation.

Our results, therefore, can be best understood in view of a reaction mechanism where there is no change in the coordination number of the active-site zinc ion on ternary complex formation, *i.e.* where substrate binding to the zinc-ion results in displacement of an inner-sphere water molecule. This would explain why ionization of zinc-bound water prevents alcohol binding to the enzyme-NAD⁺ complex. The pK_a -values (≤ 6.6) which we have assigned to zinc-bound alcohol in the enzyme-NAD⁺-alcohol complexes are consistent with a displacement mechanism and with the pK_a -value (7.6) observed for zinc-bound water in the enzyme-NAD⁺ complex. The relative magnitude of the alcohol and water pK_a -values can be explained in terms of a more extensive NAD⁺-induced charge delocalization in the metal-bound alcoholate ion than in the metal-bound hydroxide ion.

The active-site zinc ion in LADH is bound by one histidine and two negatively charged cysteine sidechains (1). This arrangement would be expected to give this zinc ion a reduced Lewis acid strength compared to that of zinc ions in enzymes where the protein provides a less negative coordination sphere. Nevertheless, the role of the active-site zinc in LADH has been discussed preferentially in terms of electrophilic catalysis of enzymic carbonyl group reduction (1). As pointed out in Paper VI, however, dehydrogenases are likely to function as bidirectional catalysts and considerable Lewis acid strength of the substrate-coordinating metal in LADH would seem to have a negative effect on the thermodynamically unfavourable process of alcohol oxidation. A hypothetical increased electrophilic character of the zinc ion can be envisaged to have effects similar to those observed in the enzyme-NAD⁺-alcohol complex for alcohol ligands having electron withdrawing substituents (*i.e.* to facilitate deprotonation of the alcohol ligand but to render hydride transfer and alcohol desorption more difficult). Considering the corresponding effects on the water and aldehyde ligands, such character of the active-site zinc ion in LADH would be disadvantageous to the processes of substrate association and dissociation in general.

With the actual arrangement of its catalytic metal center, the active-site in LADH seems most adequately designed to fulfil the catalytic function attributed to this enzyme. The pK_a -values of water and substrate alcohols, when coordinated by the active-site zinc ion in the enzyme- NAD^+ complexes, are slightly above and well below physiological pH respectively. This pK_a -difference provides the major driving force for solvent/substrate exchange at the active-site metal during alcohol oxidation. The delicate pK_a -adjustments ensure rapid alcohol binding and subsequent formation of the alcoholate ion, the active species for catalysis, without posing major obstacles to the reverse process of aldehyde reduction.

Acknowledgement

Finally, I wish to express my gratitude to professor Gösta Pettersson for his continuous support, encouragement and invaluable advice given.

Thank You Gösta !

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Kinetic Transients in the Reduction of Aldehydes Catalysed by Liver Alcohol Dehydrogenase

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The transient-state kinetics of enzymic reduction of acetaldehyde and benzaldehyde by NADH, catalyzed by horse liver alcohol dehydrogenase, have been examined under single-turnover conditions, obtained by carrying out reactions either with limiting amounts of enzyme in the presence of 20 mM pyrazole or with limiting amounts of substrate. Analysis of the variation with substrate, coenzyme, and enzyme concentrations of amplitudes and time constants for the exponential transients observed at 328 nm and 300 nm shows that the kinetics of enzymic aldehyde reduction are qualitatively and quantitatively consistent with the relationships derived in the preceding paper for an ordered ternary-complex mechanism involving identical and independent catalytic sites. It is concluded that there is no evidence whatsoever for the kinetic significance of a half-of-the-sites reactivity or any other kind of subunit interaction in the liver alcohol dehydrogenase system. The biphasic transients observed at 328 nm for the reduction of aromatic aldehydes such as benzaldehyde are a normal kinetic characteristic of the ordered ternary-complex mechanism, being attributable to accumulation of the ternary enzyme · NAD · product complex when product dissociation from this complex is slow in comparison to its formation by ternary-complex interconversion.

Horse liver alcohol dehydrogenase, which reversibly catalyzes the reduction of various aldehydes by NADH, is a dimer which two structurally identical subunits [1,2]. Classical steady-state kinetic investigations have established that the enzyme operates by an effectively ordered ternary-complex mechanism with coenzyme binding preceding the binding of substrate [3–5], and these kinetic experiments, as well as equilibrium and stopped-flow kinetic studies of coenzyme binding [6–9], have failed to reveal any cooperativity or other source of induced or pre-existing non-equivalence between the two subunits. Similarly, transient-state kinetic data for acetaldehyde-ethanol catalysis seem to be consistent with the idea that the two subunits are kinetically identical [10].

On the other hand, Bernhard *et al.* have reported stopped-flow kinetic data showing that the liver-alcohol-dehydrogenase-catalyzed reduction of various aromatic aldehydes [11] or aldehyde analogues [12] occurs in two distinct steps under single-turnover conditions with limiting amounts of substrate or coenzyme. They found that each of these steps corresponded to product formation in approximately equal amounts, and it was concluded that the two

active sites in the enzyme become kinetically non-equivalent during catalysis. The biphasic transient-state kinetic pattern was later shown to persist also when substrate and coenzyme are present in large excess to enzyme [13], and additional kinetic evidence in support of a subunit non-equivalence leading to a 'half-of-the-sites' reactivity of the enzyme during catalysis has been reported by Dunn [14] and Luisi *et al.* [15,16].

An alternative explanation for the transient-state kinetic behaviour of liver alcohol dehydrogenase has been suggested by Holbrook *et al.* [17] and by Hijazi *et al.* [18], who pointed out that ternary-complex systems under certain conditions may exhibit biphasic transients also when reactions occur at a single catalytic site. The kinetic analysis of Hijazi *et al.*, in its turn, has been criticized by Hardman *et al.* [19], who concluded that it may be necessary to assume kinetic non-equivalence of the subunits, as originally suggested. During the course of the present investigation, a further two reports considering the kinetic equivalence of the active sites of liver alcohol dehydrogenase have appeared, one concluding that the enzyme does not exhibit any half-of-the-sites reactivity [20] and one stating that the case for a half-of-the-sites reactivity has been strengthened [21].

Enzyme. Liver alcohol dehydrogenase or alcohol : NAD oxidoreductase (EC 1.1.1.1).

The above disagreement about the kinetic significance of subunit interactions in the liver alcohol dehydrogenase system appears to be due mainly to the complexity of the transient-state kinetic theory; as stated by several authors [15, 16, 22], a detailed interpretation of the results obtained has not previously been possible due to lack of a generalized analysis of the transients corresponding to an ordered ternary-complex mechanism. We have now used the relationships derived in the preceding paper to test quantitatively whether the transient-state kinetic behaviour of liver alcohol dehydrogenase can be accounted for by the classical compulsory-order mechanism, or does require the assumption of a subunit non-equivalence.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase, obtained from Boehringer Mannheim GmbH (Mannheim, Germany), was dissolved in a minimal volume of 0.05 M Tris-HCl buffer pH 8.4. After centrifugation, the enzyme was recrystallized by stepwise dialysis against increasing concentrations of ethanol [23]. Fractions precipitating at ethanol concentrations below 10% (v/v), consisting largely of the EE isomer [24], were pooled, dissolved in 0.05 M pyrophosphate buffer pH 8.8, and dialyzed for 25 h against the same buffer. Enzyme concentrations were determined spectrophotometrically by titration with NAD in the presence of pyrazole [25], and are reported throughout as active-site concentrations.

NADH (Sigma, grade III), aldehyde substrates, and pyrazole were commercial preparations of highest available purity. Aldehydes were distilled at reduced pressure under nitrogen immediately before use.

Kinetic Measurements

All experiments reported in the present investigation were performed at 25 °C in 0.05 M pyrophosphate buffer pH 8.8. Steady-state kinetic parameters for the enzymic reduction of acetaldehyde and benzaldehyde by NADH were determined spectrofluorimetrically as described previously [26]. Transient-state kinetic measurements were made by stopped-flow techniques, the oxidation of NADH being followed at the isosbestic point (328 nm) for free and enzyme-bound NADH [27]. The rate of formation of the enzyme · NAD · pyrazole complex was observed at 300 nm [13].

The transient-state kinetics of reduction of aldehyde substrates by NADH were studied either using enzyme and NADH in excess to substrate, or using substrate and NADH in excess to enzyme. In the

former case, a buffer solution of the substrate (about 16 μ M) was reacted in the stopped-flow apparatus with buffer solutions containing varied concentrations of enzyme (20–160 μ M, active-site concentration) premixed with NADH (160 μ M). In the latter case, the pyrazole catalytic-suicide method of McFarland *et al.* [13] was used to prevent cycling of the enzyme, the effect of NADH on transient reaction rates being determined by mixing the enzyme solution (about 16 μ M) with a solution containing 40 mM pyrazole, 20 mM acetaldehyde, and varied concentrations of NADH (20–200 μ M). For examination of the effect of substrate concentration on reaction rates, the enzyme solution (about 20 μ M) was premixed with NADH (usually 120 μ M) and reacted with a buffer solution containing 40 mM pyrazole and varied amounts of the aldehyde substrate (0.02–20 mM). Under such conditions, the steady-state turnover rate was found to be less than 0.01 min⁻¹ with both substrates tested, too small to have any detectable influence on the transient phase of the reaction.

The above data refer to concentrations of solutions before mixing in the stopped-flow apparatus. Values stated in the following sections refer to concentrations after mixing, *i.e.* at the start of the actual reaction.

Rapid-Reaction Equipment

Transient-state kinetic experiments were carried out with an Aminco-Morrow stopped-flow apparatus combined to a Beckman DU monochromator, an Aminco high-performance kinetic photometer, and a Kepco ABC-1000 high-voltage power supply for the photomultiplier tube. Photomultiplier signals were visualized in a Tektronix 5103N storage oscilloscope equipped with a 5A18N amplifier and a 5B10N time base, and were registered and digitalized in a Datalab DL 905 transient recorder. Apparently single-exponential transients were usually digitalized at 1024 equidistant sample points, distributed over a period in time covering at least 95% of the observed transmittance changes. For the examination of biphasic transients, sampling was performed over two different time scales, 400–600 equidistant sample points being registered within each phase. Registered data were (after analogue conversion) written out on a Hewlett-Packard 7005 B XY recorder equipped with a 17108 AM time base, or were read into a CompuCorp 425 G computer for statistical evaluation.

Data Processing

Transient-state kinetic data were analyzed in view of Eqn (1), describing the approach of the absorbance A at time t towards the final absorbance level A_{∞} in terms of two exponential transients with amplitudes A_1 and A_2 and time constants τ_1 and τ_2 , or in view of

the single-exponential relationship obtained by putting $A_2 = 0$ in Eqn (1).

$$A = A_\infty + A_1 e^{-r_1 t} + A_2 e^{-r_2 t} \quad (1)$$

Preliminary estimates of the kinetic parameters in Eqn (1) were determined by standard graphical procedures [20], using plots of $\log[A - A_\infty]$ vs t to obtain A_1 and r_1 referring to the slowest transient. When deviations from linearity were observed in such plots, parameter estimates referring to the rapid second transient were determined from secondary plots of $\log[A - A_\infty - A_1 e^{-r_1 t}]$ vs t .

Final parameter estimates were calculated statistically, using the non-linear regression methods routinely employed in our laboratories for the analysis of kinetic data [28]. Correction terms for the preliminary parameter estimates were thus calculated at a pre-determined level of self-consistency (usually 0.001%) by iterative least-squares fitting of the regression function given in Eqn (1) to the experimental data, after primary conversion of registered transmittances into absorbances. In cases where contributions from a second transient were found to be statistically insignificant, the single-exponential regression function obtained by putting $A_2 = 0$ in Eqn (1) was fitted to the experimental observations.

The experimental time-scale defined by the stop of the flow was corrected for a dead-time of 5 ms of the stopped-flow apparatus, determined as described by Gutfreund [29]. Transient-state kinetic parameters plotted in Fig. 2–6 and 9 represent means of 2–4 separate determinations.

RESULTS

Steady-State Kinetic Experiments

Kinetic parameters referring to the Dalziel rate equation [5]

$$\frac{c_E}{v} = \phi_0 + \frac{\phi_1}{[R]} + \frac{\phi_2}{[S]} + \frac{\phi_{12}}{[R][S]} \quad (2)$$

for the steady-state molar reaction velocity (v/c_E) of the liver alcohol dehydrogenase catalyzed reduction of acetaldehyde and benzaldehyde (S) by NADH (R) were determined by standard spectrofluorimetric techniques. The results obtained are listed in Table 1, and agree well with Michaelis-Menten parameters for the corresponding reactions reported previously [4, 11, 12].

Transient-State Kinetics of Acetaldehyde Reduction

As has previously been reported for the corresponding reaction at pH 7 [10], reduction of acetaldehyde by NADH at pH 8.8 in the presence of limiting amounts of liver alcohol dehydrogenase was found to result in an apparent first-order decrease of the

Table 1. Steady-state kinetic parameters for the enzymic reduction of acetaldehyde and benzaldehyde by NADH. Parameters refer to Eqn (2), and were determined spectrofluorimetrically at 25 °C in 0.05 M pyrophosphate buffer pH 8.8

Substrate	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}
	s	$\mu\text{M s}$		$\mu\text{M}^2 \text{s}$
Acetaldehyde	0.10 (± 0.01)	0.20 (± 0.02)	19 (± 2)	12 (± 5)
Benzaldehyde	0.18 (± 0.02)	—	4.2 (± 0.5)	—

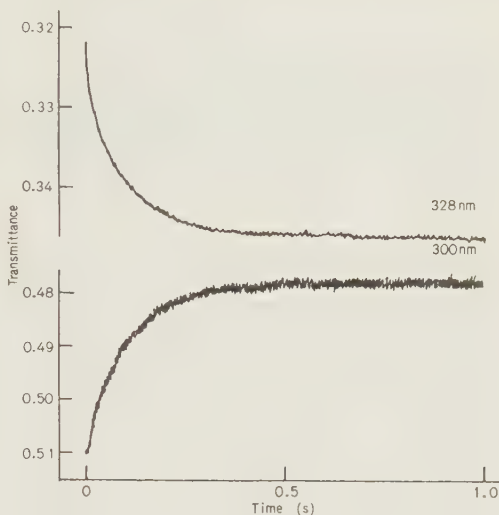


Fig. 1. Transient reduction of acetaldehyde by NADH in the presence of limiting amounts of liver alcohol dehydrogenase, followed at 328 and 300 nm. 200 μM acetaldehyde and 100 μM NADH in 0.05 M pyrophosphate buffer pH 8.8, containing 20 mM pyrazole and 8.9 μM enzyme

absorbance at 328 nm, attributable to NADH disappearance (Fig. 1). The amplitude and time constant for this single-exponential transient was determined as a function of substrate and coenzyme concentrations, using the catalytic-suicide method of McFarland *et al.* [13] to prevent cycling of the enzyme. Reactions were thus carried out in the presence of 20 mM pyrazole, under which condition there is a rapid conversion of the enzyme · NAD complex formed during catalysis into a non-productive enzyme · NAD · pyrazole complex. Fig. 1 illustrates that formation of the latter complex, as determined from the increase in absorbance at 300 nm, occurs concomitantly with the oxidation of NADH followed at 328 nm. The increase in absorbance at 300 nm, also, was found to conform to apparent first-order kinetics, apart possibly from an initial lag phase lasting for less than 10 ms.

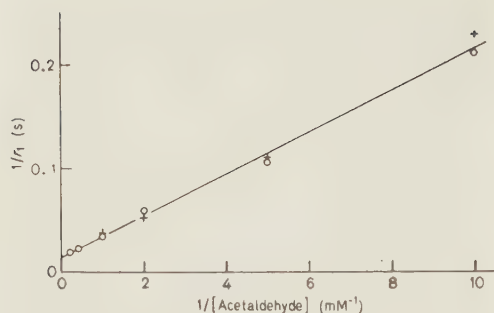


Fig. 2. Dependence on substrate concentration of the transient time constant τ_1 for the enzymic reduction of acetaldehyde by NADH. Time constants were determined from absorbance changes recorded at 328 nm (O) or 300 nm (+) for the reduction of varied amounts of benzaldehyde by 100 μ M NADH in 0.05 M pyrophosphate buffer pH 8.8, containing 20 mM pyrazole and 8.9 μ M liver alcohol dehydrogenase

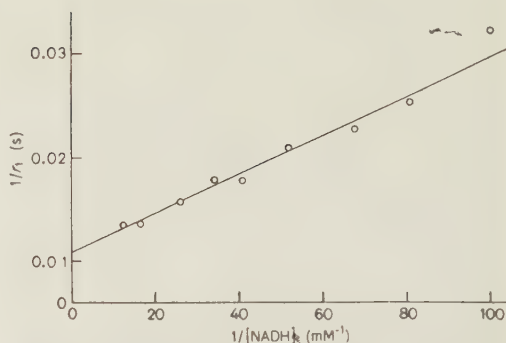


Fig. 3. Dependence on NADH concentration of the transient time constant τ_1 for the enzymic reduction of acetaldehyde by NADH. Time constants were determined from absorbance changes recorded at 328 nm for the reduction of 10 mM acetaldehyde by varied amounts of NADH in 0.05 M pyrophosphate buffer pH 8.8, containing 20 mM pyrazole and 7.6 μ M liver alcohol dehydrogenase

Amplitudes of the exponential transients observed at 328 and 300 nm, *i.e.* recorded absorbance changes extrapolated to zero reaction time, did not vary significantly with the concentration of substrate or coenzyme, although up to 30% of the absorbance changes were found to occur within the dead-time of the stopped-flow apparatus at the highest reactant concentrations tested. According to the difference absorption coefficients at the above wavelengths reported previously [16], amplitudes were found to correspond well to disappearance of NADH and appearance of the enzyme $\cdot$ NAD $\cdot$ pyrazole complex in amounts agreeing with the active-site concentration of enzyme, confirming that the enzymic reaction in the presence of 20 mM pyrazole is limited essentially to a single turnover on the time scale examined in the transient-state kinetic experiments [13].

Transient time constants τ_1 calculated from the 328-nm absorbance changes were found to agree (within an experimental precision of about 10%) with those obtained from the 300-nm absorbance changes, and exhibited a pronounced dependence on substrate and coenzyme concentrations. Fig. 2 shows a double-reciprocal plot for the variation of τ_1 with substrate concentration at a fixed high concentration of NADH (100 μ M). Experimental points in this plot fall well along a straight line with an intercept corresponding to a limiting τ_1 value of $80 (\pm 15) \text{ s}^{-1}$ and a slope of $20 (\pm 1) \mu\text{M s}$. The latter values agrees excellently with the magnitude of the kinetic parameter ϕ_2 determined for the steady-state enzymic reduction of acetaldehyde by NADH (see Table 1).

The dependence of τ_1 on NADH concentration was determined using 10 mM acetaldehyde and 15–100 μ M NADH. As shown in Fig. 3, experimental

points in double-reciprocal plots of the latter data fall well along a straight line with an intercept corresponding to a limiting rate of $90 (\pm 10) \text{ s}^{-1}$, not significantly different from that calculated for the data in Fig. 2. The slope of the regression line in Fig. 3 was found to be $0.18 (\pm 0.02) \mu\text{M s}$, closely agreeing with the value of the kinetic parameter ϕ_1 for the corresponding steady-state reaction (Table 1).

Transient-State Kinetics of Benzaldehyde Reduction with Limiting Amounts of Enzyme

The transient-state kinetics at pH 8.8 for the enzymic reduction of benzaldehyde by NADH in the presence of limiting amounts of liver alcohol dehydrogenase were examined at a fixed high concentration of coenzyme, using the above catalytic-suicide method to obtain single-turnover conditions. As has been well established for this reaction [13,21,30], the decrease in absorbance at 328 nm during catalysis under such conditions is governed by two exponential transients, amplitudes (A_i) and time constants (τ_i) of which were determined for a series of concentrations of benzaldehyde (0.02–20 mM). At the highest substrate concentrations tested, up to 70% of the total absorbance changes were found to occur within the dead-time of the stopped-flow apparatus, and reliable direct estimates of parameters for the fast transient could be obtained only at benzaldehyde concentrations below 1 mM.

In consistence with previous reports, the sum $A_1 + A_2$ of amplitudes for the two transients was found to be essentially independent of substrate concentration, corresponding well to NADH oxidation in amounts equal to the active-site concentration of

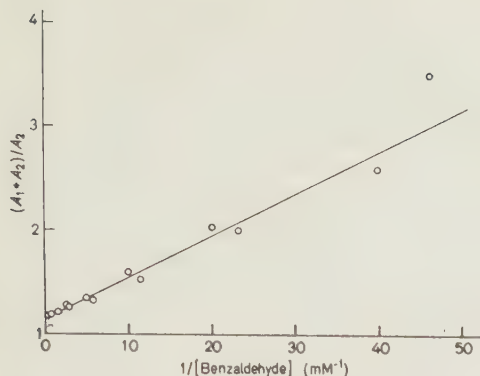


Fig. 4. Dependence on substrate concentration of the amplitude A_2 of the fast 328-nm transient for the enzymic reduction of benzaldehyde by *NADH*. The relative magnitude of A_2 in comparison to the total absorbance change ($A_1 + A_2$) was determined in 0.05 M phosphate buffer pH 8.8, containing 9.3 μ M liver alcohol dehydrogenase, 20 mM pyrazole, 60 μ M *NADH*, and varied amounts of substrate

enzyme. The relative contributions of the two transients vary considerably, however, such that the amplitude A_2 of the fast transient steadily increases with increasing concentrations of benzaldehyde. This is shown by the double-reciprocal plot in Fig. 4, which also indicates that the relative magnitude of A_2 is hyperbolically dependent on substrate concentration. The regression line for the data in Fig. 4 has a slope of $40 (\pm 8) \mu$ M and an intercept slightly larger than unity (1.15 ± 0.05), the latter value indicating that the slow transient cannot be completely depressed by excess substrate. This might partly reflect minor differences between absorption coefficients for the ternary enzyme $\cdot$ *NAD* $\cdot$ product and enzyme $\cdot$ *NAD* $\cdot$ pyrazole complexes, and will not be further commented upon due to lack of sufficiently detailed knowledge about the actual absorption coefficients [20].

Fig. 5 and 6 show double-reciprocal plots for the dependence on substrate concentration of time constants for the slow (r_1) and the fast (r_2) transient, respectively. Data in Fig. 5 conform to a regression line with an intercept corresponding to a limiting r_1 value of $10 (\pm 2) \text{ s}^{-1}$. The slope of the line is $4.5 (\pm 0.8) \mu$ M s, agreeing well with the ϕ_2 value determined for the steady-state reduction of benzaldehyde (Table 1). The curve drawn in Fig. 6 represents a least-squares fit of the regression function

$$\frac{1}{r_2} = \frac{1}{r_{2,\infty}} \cdot \frac{[S] + K_{app}}{[S] + \phi_2 r_{1,\infty}} \quad (3)$$

using $\phi_2 r_{1,\infty} = 45 \mu$ M, and corresponds to the best-fit estimates $r_{2,\infty} = 320 (\pm 90) \text{ s}^{-1}$ and $K_{app} = 400 (\pm 150) \mu$ M.

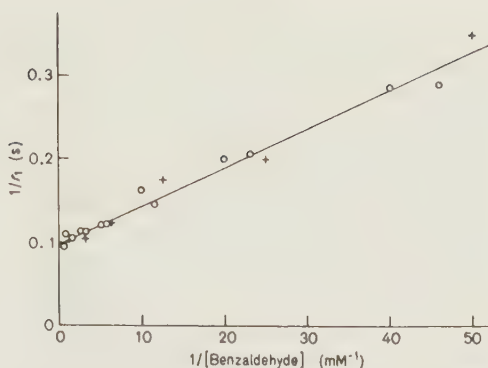


Fig. 5. Dependence on substrate concentration of the time constant r_1 of the slow 328-nm transient for the enzymic reduction of benzaldehyde by *NADH*. Conditions as in Fig. 4. Included are values of r_1 determined from the transient absorbance changes at 300 nm (+)

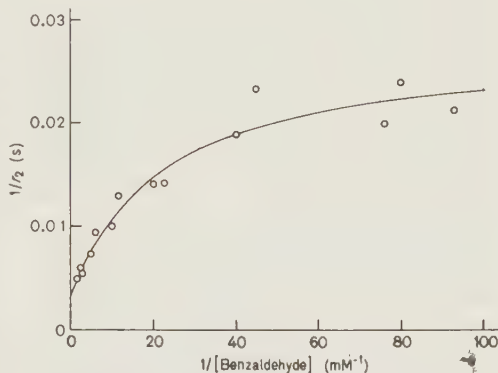


Fig. 6. Dependence on substrate concentration of the time constant r_2 of the fast 328-nm transient for the enzymic reduction of benzaldehyde by *NADH*. Conditions as in Fig. 4

Formation of the enzyme $\cdot$ *NAD* $\cdot$ pyrazole complex during catalysis under the above conditions was followed at 300 nm for some selected concentrations of benzaldehyde. As indicated by the typical result shown in Fig. 7, the total increase in absorbance at 300 nm was invariably found to correspond well to the active-site concentration of enzyme. Fig. 7 further illustrates that the 300-nm absorbance, after an initial lag period coinciding in time with the fast transient observed at 328 nm, increases at a rate corresponding to that of the slow transient observed at 328 nm. Time-constants calculated from first-order plots of the 300-nm absorbance changes agreed well with those obtained for the slow 328-nm transient (see Fig. 5).

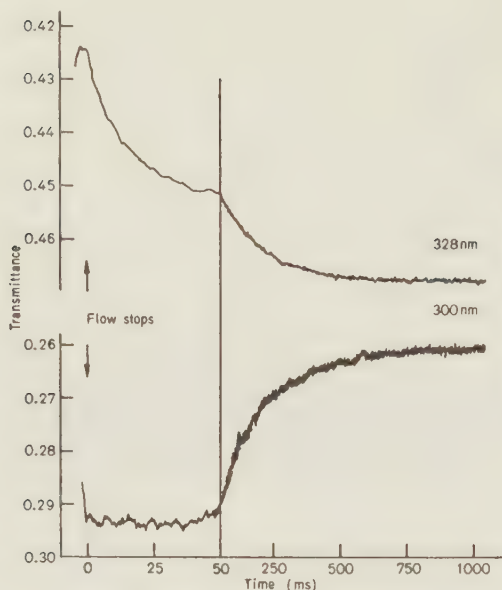


Fig. 7. Transient reduction of benzaldehyde by NADH in the presence of limiting amounts of liver alcohol dehydrogenase, followed at 328 and 300 nm. Conditions as in Fig. 4, using 83 μM substrate. The time scale has not been corrected for the dead-time of the stopped-flow apparatus

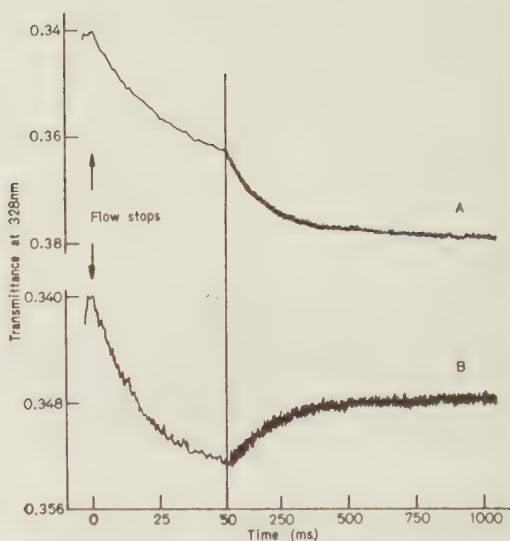


Fig. 8. Transmittance changes at 328 nm during the enzymic reduction of limiting amounts of benzaldehyde by NADH. 9.5 μM benzaldehyde, 80 μM NADH, and 72 μM liver alcohol dehydrogenase in 0.05 M pyrophosphate buffer pH 8.8, in the absence (A) or presence (B) of 5 mM ethanol. The time scale has not been corrected for the dead-time of the stopped-flow apparatus

Transient-State Kinetics of Benzaldehyde Reduction with Limiting Amounts of Substrate

In order to examine an alternative experimental approach for the study of kinetic transients, the enzymic reduction of benzaldehyde by NADH was followed at 328 nm using limiting amounts of substrate to prevent cycling of the enzyme. As reported previously [11,16] and illustrated by Fig. 8, the bi-phasic kinetic pattern persists under such conditions, and amplitudes and time constants for the two transients were determined at a fixed high concentration of NADH for a series of enzyme concentrations intermediate between the concentrations of substrate and coenzyme. The results thus obtained over the fairly narrow concentration range available for experimental variation (20–80 μM enzyme active-site) appeared to be entirely analogous to those described for the above catalytic-suicide experiments, *i.e.* the dependence of amplitudes and time constants of the two transients on enzyme active-site concentration was found not to differ significantly from the dependence on substrate concentration established for the catalytic-suicide reaction with limiting amounts of enzyme. This is shown in Fig. 9 by example of the double-reciprocal plot obtained for the variation of τ_1 with the active-site concentration of enzyme.

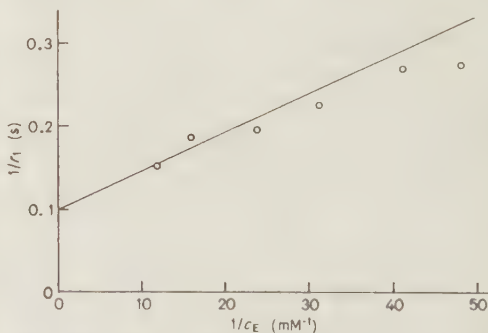


Fig. 9. Dependence on enzyme concentration of the time constant τ_1 of the slow 328-nm transient for the enzymic reduction of benzaldehyde by NADH. 100 μM NADH, 8 μM benzaldehyde, and varied active-site concentrations of liver alcohol dehydrogenase (C_E) in 0.05 M pyrophosphate buffer pH 8.8

Experimental points in this plot fit satisfactorily well to a line with the same slope and intercept as the regression line calculated for the data in Fig. 5.

The above reactions were also carried out in the presence of 5 mM ethanol, which causes a rapid conversion of the binary enzyme · NAD complex formed during the catalytic reduction of benzaldehyde into

the ternary enzyme · NAD · ethanol complex, resulting in a rapid reversal of the process of NADH oxidation through reduction of NAD by ethanol [10]. As shown in Fig. 8, the amplitude of the slow transient observed at 328 nm changes sign under such conditions, whereas the amplitude of the fast transient and time constants for both transients remain essentially unaffected. This observation provides direct evidence that the slow transient can be attributed to formation of the enzyme · NAD complex, *i.e.* to product dissociation from the ternary enzyme · NAD · benzylalcohol complex, and also indicates that there is a transient accumulation of the latter complex during the catalytic reduction of benzaldehyde under single-turnover conditions.

DISCUSSION

Assuming that liver alcohol dehydrogenase operates by the ordered ternary-complex mechanism



where E, S, P, R, and O denote enzyme, substrate, product, NADH, and NAD, respectively, absorbance changes at 328 nm recorded for the catalytic reduction of aldehydes by NADH in the presence of 20 mM pyrazole reflect formation of the enzymic complexes EOP and EO, the latter being rapidly trapped as an abortive enzyme · NAD · pyrazole complex. As reported in the preceding paper, the analytical solution of the kinetic differential equations corresponding to the above reaction mechanism prescribes that the concentration of (trapped) EO steadily increases with time (*t*) in accordance with the single-exponential relationship

$$\frac{[EO]}{c_E} = 1 - e^{-\tau_1 t} \quad (4)$$

where c_E denotes the active-site concentration of enzyme, and where τ_1 is defined by

$$\frac{1}{\tau_1} = \frac{1}{\tau_{1,\infty}} + \frac{\phi_1}{[R]} + \frac{\phi_2}{[S]} + \frac{\phi_{12}}{[R][S]} \quad (5)$$

The concentration of EOP, on the other hand, passes through a maximum, being governed by two exponential transients ($\tau_2 > \tau_1$):

$$\frac{[EOP]}{c_E} = \frac{\tau_1}{k'_{-2}} (e^{-\tau_1 t} - e^{-\tau_2 t}) \quad (6)$$

When formation of EOP from ERS is slow in comparison to the conversion of EOP into ERS or into EO and product ($k \ll k' + k'_{-2}$), no significant

amounts of EOP accumulate during catalysis, and the decrease in absorbance at 328 nm can be attributed mainly to formation of the trapped binary complex EO. Such conditions undoubtedly prevail as concerns the enzymically catalyzed reduction of acetaldehyde by NADH, for which reaction a single-exponential transient with an amplitude agreeing with the active-site concentration of enzyme is observed at 328 nm, coinciding with the transient reflecting formation of the trapped EO complex observed at 300 nm. According to the steady-state kinetic data listed in Table 1, contributions from the ϕ_{12} term in Eqn (5) to the magnitude of the time constant τ_1 for this transient are negligible (less than 3%) over the ranges of substrate and coenzyme concentrations (above 10 μ M) accessible for variation in the present transient-state kinetic experiments. Eqn (5), therefore, prescribes that double-reciprocal plots for the dependence of τ_1 on concentrations of substrate and coenzyme should yield straight lines with slopes equal to ϕ_2 and ϕ_1 , respectively. The results shown in Fig. 2 and 3 provide clear evidence that such is the case, which establishes the validity of the relationships derived in the preceding paper and confirms the previous conclusion that the transient-state kinetics of acetaldehyde reduction are consistent with an ordered ternary-complex mechanism [10].

When relationships between rate constants in the above ordered ternary-complex mechanism are such ($k \gg k' + k'_{-2}$) that formation of EOP can be rapid in comparison to its breakdown, detectable amounts of EOP may accumulate during catalysis. Absorbance changes recorded at 328 nm may then exhibit a biphasic transient rate behaviour, due to simultaneous contributions from the fast transient governing the rate of formation of EOP according to Eqn (6) and the slow transient reflecting the ultimate formation of the trapped EO complex. The rate of formation of EOP, and hence accumulation of this complex and contributions from the fast 328-nm transient, would obviously be expected to increase with increasing concentrations of substrate and coenzyme.

Transient-state kinetic results for the enzymic reduction of benzaldehyde by NADH are qualitatively consistent with such a mechanism. Firstly, two transients are observed at 328 nm, the relative contributions of which exhibit the expected variation with substrate concentration at a fixed high concentration of coenzyme. Secondly, it has been inferred previously from the study of isotope effects on the kinetics of the reaction that the fast 328-nm transient reflects ternary-complex isomerization, and that the slow transient can be attributed to product dissociation from EOP [11,13]. The latter conclusion is strongly supported by the present observation that formation of the trapped EO complex, measured at 300 nm, occurs mainly in a single-exponential process coinciding with the slow

328-nm transient, the initial lag phase in absorbance changes at 300 nm being attributable to the progressive increase in concentration of EOP reflected by the rapid 328-nm transient. Finally, the experiments carried out with limiting amounts of substrate in the presence of a large excess of ethanol provide additional evidence that the slow transient reflects product dissociation from EOP, and clearly indicate that the concentration of EOP (the main NAD-containing species observed under such conditions) in accordance with Eqn (6) passes through a maximum determined by a fast transient representing formation of EOP and a slow transient representing breakdown of EOP. In other words, the data in Fig. 8 convincingly demonstrate that the fast 328-nm transient can be attributed to accumulation of EOP.

The transient-state kinetics of benzaldehyde reduction by a fixed high concentration of NADH can, in fact, be quantitatively accounted for in view of the relationships derived for the ordered ternary-complex mechanism under the assumption that $k \gg k' + k'_{-2}$. The slope of the straight line obtained in double-reciprocal plots for the variation with substrate concentration of the time constant r_1 referring to the slow transient (Fig. 5) thus agrees with the ϕ_2 value for the corresponding steady-state reaction, as predicted by Eqn (5). The intercept of the line corresponds to $r_{1,\infty} = 10 \text{ s}^{-1}$, which means that $\phi_2 r_{1,\infty} \approx 45 \mu\text{M}$ (Table 1). As predicted by the theory, the latter value agrees well with that (40 μM) obtained for the slope of the regression line in Fig. 4, describing the dependence of amplitudes on substrate concentration. The variation of r_2 with substrate concentration, similarly, conforms well with Eqn (3), which represents the functional relationship derived in the preceding paper for the time constant of the fast transient. It may be observed that Eqn (3) at high concentrations of substrate reduces to

$$\frac{1}{r_2} = \frac{1}{r_{2,\infty}} \left(1 + \frac{K_{\text{app}}}{[S]} \right) \quad (7)$$

which is identical with the empirical relationship previously reported to govern the rate of the fast transient in the enzymic reduction of aromatic aldehydes [13,21,30].

Evaluation of intercepts calculated for the regression lines in Fig. 2 and 3 in terms of Eqn (5) yields an estimate of $r_{1,\infty}$ approximately equal to 100 s^{-1} for the enzymic reduction of acetaldehyde, whereas the $r_{1,\infty}$ value reported above for benzaldehyde reduction is 10 s^{-1} . As was shown in the preceding paper, the transient-state kinetic parameter $r_{1,\infty}$ should be related to the steady-state kinetic parameter ϕ_0 through

$$\phi_0 = \frac{1}{r_{1,\infty}} + \frac{1}{k'_{-1}} \quad (8)$$

in an ordered ternary-complex mechanism. Using the above estimates of $r_{1,\infty}$ and the ϕ_0 values listed in Table 1, experimental data for acetaldehyde and benzaldehyde reduction yield consistent estimates of k'_{-1} according to Eqn (8) (11 and 12 s^{-1} , respectively), closely agreeing with the value $k'_{-1} \approx 13 \text{ s}^{-1}$ determined previously by direct measurement of the rate of coenzyme-dissociation from EO [14]. The present experimental results are thus consistent with the basic kinetic relationship expressed in Eqn (8), also, and provide direct evidence that the rate of formation of EO is partially rate-limiting in the steady-state reduction of benzaldehyde, whereas reduction of acetaldehyde during steady-state is rate-limited mainly by the final step of coenzyme dissociation.

Summing up the above discussion, it may be concluded that the present kinetic data for the enzymic reduction of two typical substrates of liver alcohol dehydrogenase are fully consistent with an ordered ternary-complex mechanism in all respects tested, the appearance of biphasic 328-nm absorbance changes during benzaldehyde reduction being attributable to transient accumulation of the ternary complex EOP. As was stated above, there cannot be any considerable accumulation of EOP unless $k \gg k' + k'_{-2}$. This means, firstly, that ternary-complex equilibration must favour the formation of EOP ($k \gg k'$). Such conditions appear to prevail with most aldehyde substrates examined up to date, which provides an explanation for the fact that biphasic transients never have been observed in the reverse reaction of enzymic alcohol oxidation by NAD. Secondly, product dissociation from EOP must be slow in comparison to the forward rate of ternary-complex isomerization ($k \gg k'_{-1}$), which appears to be the critical factor deciding whether or not a biphasic 328-nm transient-rate behaviour is obtained with different substrates. The results obtained with benzaldehyde illustrate the kinetic behaviour of the liver alcohol dehydrogenase system when product dissociation from EOP is slow, and are in all essential features typical for the biphasic transient-state kinetics observed with other aromatic aldehydes or aldehyde analogues.

The present results seem to establish the validity of the theoretical analysis given in the preceding paper, which provides adequate relationships for the interpretation of transient-state kinetic parameters such as $r_{1,\infty}$, $r_{2,\infty}$, and K_{app} in terms of individual rate constants in the reaction mechanism. Such an evaluation of the kinetic results is beyond the scope of the present investigation, but it should be pointed out that the above parameters are all composite constants, which cannot be uncritically identified with the rate constant associated with a single reaction step in the mechanism. It may be of particular importance to recognize this fact in the study of isotope or substituent effects on the kinetic parameters, where effects observed

might be due to a large variation of a rate constant not normally contributing much to the magnitude of the parameter.

The initial suggestion that liver alcohol dehydrogenase exhibits a half-of-the-sites reactivity was put forward in order to explain the biphasic absorbance changes at 328 nm occurring during the enzymic reduction of aromatic aldehydes, and the biphasic transient rate behaviour of the system still represents the main evidence presented in support of this suggestion; early interpretations of certain spectrometric data for ligand binding to the enzyme in terms of subunit interactions [31, 32] have later been modified and the data explained in terms of identical sites [8, 17, 33]. The present investigation shows that even the transient-state kinetic behaviour of the system is fully consistent with an ordered ternary-complex mechanism involving identical and independent sites. The occurrence of biphasic 328-nm transients during the reduction of aldehyde substrates yielding slowly dissociating products is an inevitable kinetic consequence of the compulsory-order mechanism, and a kinetic non-equivalence of the enzyme subunits would result in a transient rate behaviour more complex than just biphasic under such conditions. In view of the data presented up to date, therefore, it may be concluded that there is no evidence whatsoever for the kinetic significance of any kind of subunit interactions in the liver alcohol dehydrogenase system, particularly not of subunit interactions leading to a half-of-the-sites reactivity of the enzyme.

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Effect of pH on Coenzyme Binding to Liver Alcohol Dehydrogenase

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1. The transient-state kinetics of ligand-displacement reactions have been analyzed. Methods based on this analysis have been used to obtain reliable estimates of on-velocity and off-velocity constants for coenzyme binding to liver alcohol dehydrogenase at different pH values between 6 and 10.

2. The rate of NADH dissociation from the enzyme shows no pronounced dependence on pH. The rate of NAD^+ dissociation is controlled by a group with a pK_a of 7.6, agreeing with the pK_a reported to regulate the binding of certain inhibitory substrate analogues to the enzyme $\cdot \text{NAD}^+$ complex.

3. Critical experiments have been performed to test a recent proposal that on-velocity constants for the binding of NADH and NAD^+ are controlled by proton equilibria exhibiting different pK_a values. The results show that association rates for NADH and NAD^+ exhibit the same pH dependence corresponding to a pK_a of 9.2. Titrimetric evidence is presented indicating that the latter effect of pH derives from ionization of a group which affects the anion-binding capacity of the coenzyme-binding site.

The catalytic activity of liver alcohol dehydrogenase is strongly dependent on pH over the pH range 6–10 [1]. It has been well established that this pH dependence derives from the combined effects of pH on several reaction steps in the catalytic mechanism; proton equilibria involving ionizing groups on the enzyme are known to affect the binding of both coenzyme and substrate, as well as interconversion of the productive ternary complexes [2–7]. These proton equilibria must form part of the enzymic reaction in such a way that they account for the net production or consumption of protons during catalysis. A detailed understanding of the enzymic dehydrogenation process, therefore, requires a detailed and coherent description of the pH dependence of each individual reaction step in the catalytic mechanism.

To arrive at such a coherent description of the effect of pH on liver alcohol dehydrogenase catalysis, two major ambiguities concerning the pH dependence of coenzyme binding have to be resolved. First, it must be clarified whether association rates for the binding of NADH and NAD^+ are dependent on proton equilibria exhibiting the same or different pK_a values. The former is suggested by the stop-flow kinetic data presented by De Traglia et al. [8], who found that on-velocity constants for the binding of NADH and NAD^+ show the same pH profile corresponding to

a pK_a of 9.5. Shore et al. [9], however, concluded from similar experiments that NADH association exhibits a pK_a 9.0 dependence distinct from the pK_a 10.0 dependence observed for NAD^+ association. These controversial results obviously lead to entirely different inferences about the mechanism of proton transfer during enzyme catalysis.

Secondly, it is of importance to clarify whether substrate binding to the enzyme $\cdot \text{NAD}^+$ complex can be assumed to be dependent on the same ionizing group as the one controlling the NAD^+ dissociation rate. This idea has been advanced by Shore et al. [10], who suggested that the pK_a 7.6 dependence observed for the binding of certain inhibitory substrate analogues reflects the pH dependence of NAD^+ dissociation. Direct off-velocity constant determinations, however, led to the conclusion that NAD^+ dissociation is dependent on a group with a significantly higher pK_a [8]. The latter observation might indicate that substrate binding and coenzyme dissociation are actually controlled by different ionizing groups.

To resolve these ambiguities we have reinvestigated the pH dependence of coenzyme binding to liver alcohol dehydrogenase by stop-flow techniques. The present paper provides an analysis of the transient-state kinetics of ligand displacement reactions, and methods based on this analysis have been applied to obtain reliable estimates of rate constants for coenzyme association and dissociation between pH 6

Enzyme. Liver alcohol dehydrogenase or alcohol: NAD^+ oxidoreductase (EC 1.1.1.1).

and 10. The results have been checked by measurement of the stoichiometry of proton release or uptake resulting from the binding of coenzyme and coenzyme analogues to the enzyme at different pH. The origin and mechanistic significance of the observed effects of pH is discussed.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer Mannheim GmbH was further purified as described by Bernhard et al. [11]. Enzyme concentrations were determined fluorimetrically by titration with NaDH in the presence of isobutyramide [12], and are reported throughout as active-site concentrations.

The coenzymes NADH and NAD⁺ were purchased from Sigma Chemical Corp. (grade III) and were used without further purification. Other reagents were of analytical grade. Buffer solutions of ionic strength 0.1 M were prepared using water twice distilled from a quartz apparatus. Experiments at pH 6–9 were performed in phosphate buffer, while bicarbonate buffer was used above pH 9.

Kinetic Measurements and Data Processing

All experiments reported in the present investigation were performed at 25°C. Transient-state kinetic measurements were made photometrically by stop-flow techniques, using the rapid-reaction equipment described previously [7]. Rate parameters for the exponential transients observed were estimated by non-linear regression analysis as detailed previously [7].

NADH Dissociation Rates

Apparent first-order rate constants for the displacement of NADH from liver alcohol dehydrogenase were determined at 297 nm by reacting the enzyme · NADH complex with varied concentrations of NAD⁺ (0.1–4 mM) in the presence of 5 mM pyrazole [8]. The enzyme · NADH complex was prepared by pre-equilibrating the enzyme (5 µM) with NADH before mixing in the stop-flow apparatus, using coenzyme concentrations calculated to give 90–95% saturation of the enzyme according to the equilibrium binding data reported by Theorell et al. [2].

NADH Association Rates

Apparent first-order rate constants for the binding of NADH to liver alcohol dehydrogenase were determined by reacting the enzyme (about 1 µM) with varied concentrations of NADH (5–150 µM) in the

presence of 50 µM *trans*-*N,N*-dimethylaminocinnamaldehyde. This aldehyde is known to combine specifically to the enzyme · NADH complex, and the aldehyde concentration used is sufficiently high to obtain a rapid equilibration with the enzyme · NADH complex over the entire pH range examined [13]. Under such conditions the binding of NADH to free enzyme can be directly monitored through the absorbance changes observed at 464 nm (the wavelength of maximum absorption of the enzyme · NADH · aldehyde complex [13]). Apparent first-order rate constants (20–200 s⁻¹) were determined for 5–7 different NADH concentrations at each fixed pH, and the corresponding second-order rate constant for NADH association was calculated by linear regression analysis. Measurements were performed over the pH range 7–10, where the rate of breakdown of the chromophoric ternary complex is small (0.01–0.5 s⁻¹ [14]) in comparison to the observed NADH association rates.

NAD⁺ Dissociation Rates

Apparent first-order rate constants for the displacement of NAD⁺ from liver alcohol dehydrogenase were determined at 360 nm by reacting the enzyme · NAD⁺ complex with varied concentrations of NADH (20–300 µM) in the presence of 1 mM isobutyramide [8]. The enzyme · NAD⁺ complex was prepared by pre-equilibrating the enzyme (8 µM) with NAD⁺ before mixing, using coenzyme concentrations calculated to give 90–95% saturation of the enzyme according to previously reported equilibrium data [2, 3]. As has been previously observed [3], pre-incubation of the enzyme with NAD⁺ led to the formation of small amounts of NADH (1–3 µM). This was taken into account on calculation of the amount of NAD⁺ required to obtain the desired concentration of the enzyme · NAD⁺ complex. The presence of 1 mM isobutyramide had no significant effect on the reaction rates observed below pH 8. At higher pH, reaction rates obtained in the presence of 1 mM isobutyramide agreed with those observed at 464 nm using 50 µM *trans*-*N,N*-dimethylaminocinnamaldehyde as a trapping agent.

NAD⁺ Association Rates

Apparent first-order rate constants for the binding of NAD⁺ to liver alcohol dehydrogenase were determined at 464 nm by reacting the enzyme (1 µM) with varied concentrations of NAD⁺ (5–50 µM) in the presence of 6 mM propanol and 50 µM *trans*-*N,N*-dimethylaminocinnamaldehyde, the latter two reagents being used to obtain a rapid conversion of the enzyme · NAD⁺ complex into the chromophoric enzyme · NADH · cinnamaldehyde-derivative complex.

Reaction rates obtained with 4 mM NAD^+ were found to exceed 300 s^{-1} over the entire pH range examined, i.e. 464-nm absorbance changes observed with NAD^+ concentrations below $50 \mu\text{M}$ can be assumed to be rate-limited only by NAD^+ association. Second-order rate constants for NAD^+ association at different pH (7–10) were calculated as described above for NADH association.

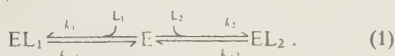
Titrimetric Measurements

The amount of protons released to or withdrawn from solution on ligand binding to liver alcohol dehydrogenase was measured with a Radiometer SBR 2 titrator equipped with a TTT 60 titrator and a PHM 64 pH meter. Measurements were performed in a closed vessel under nitrogen to prevent pH changes due to carbon dioxide uptake. Enzyme preparations used for the titrimetric experiments were dialyzed under nitrogen at 4°C against 33 mM sodium sulphate (pH 9.5). Reaction solutions contained 80 nmol enzyme and were adjusted to the appropriate pH with dilute NaOH or HCl. Ligands, dissolved in 33 mM sodium sulphate solutions adjusted to the same pH (± 0.02 unit), were then added and the amount of HCl or NaOH required to restore the original pH of the reaction solution was recorded.

RESULTS

The Transient-State Kinetics of Displacement Reactions

Eqn (1) shows the reaction scheme for a competitive binding of two ligands (L_1 and L_2) to an enzyme (E):



The corresponding kinetic equations are given by

$$\frac{d[\text{EL}_i]}{dt} = k_i [\text{L}_i] [\text{E}] - k_{-i} [\text{EL}_i]; \quad i = 1, 2 \quad (2)$$

$$c_i = [\text{L}_i] + [\text{EL}_i]; \quad i = 1, 2 \quad (3)$$

$$c_E = [\text{E}] + [\text{EL}_1] + [\text{EL}_2] \quad (4)$$

where c_i and c_E denote total concentrations of the respective reactants. In a typical displacement experiment the enzyme is pre-equilibrated with a sufficiently high concentration of one ligand (say L_1) to ensure that EL_1 is the predominant enzyme species at zero reaction time. The reaction is then initiated by adding L_2 in amounts large enough to cause an extensive displacement of L_1 through formation of the species EL_2 , i.e. we have

$$\frac{k_2 [\text{L}_2]}{k_{-2}} \gg \frac{k_1 [\text{L}_1]}{k_{-1}} > 1. \quad (5)$$

The displacement reaction is usually followed by monitoring changes in the concentration of either EL_1 or EL_2 . The time dependence of these concentration variables is governed by the non-linear differential equations in Eqn (2), to which there is no general analytical solution. Assuming that both ligands are present in large excess to enzyme ($c_i \gg c_E$), however, the approximations $[\text{L}_i] \approx c_i$ are valid and may be used for linearisation of Eqn (2). Under such conditions, Eqns (2–4) can be solved by standard methods of linear algebra [15] to give

$$[\text{EL}_i] = A_{0i} + A_{1i} e^{-r_1 t} + A_{2i} e^{-r_2 t}; \quad i = 1, 2 \quad (6)$$

where r_1 and r_2 are roots of the characteristic equation

$$r^2 - (k_{-1} + k_1 [\text{L}_1] + k_{-2} + k_2 [\text{L}_2]) r + k_{-1} k_{-2} + k_{-1} k_2 [\text{L}_2] + k_{-2} k_1 [\text{L}_1] = 0. \quad (7)$$

The exact expressions for r_1 and r_2 obtained by solution of Eqn (7) will not be considered here. The two roots will be real and are likely to differ considerably in magnitude ($r_2 \gg r_1$) in all cases of practical interest, and are then given approximately [15] by

$$r_2 = k_{-1} + k_1 [\text{L}_1] + k_{-2} + k_2 [\text{L}_2] \quad (8)$$

$$r_1 = \frac{k_{-1} k_{-2} + k_{-1} k_2 [\text{L}_2] + k_{-2} k_1 [\text{L}_1]}{k_{-1} + k_1 [\text{L}_1] + k_{-2} + k_2 [\text{L}_2]}. \quad (9)$$

When the relationships in Eqn (5) are valid, Eqn (9) can be reduced and written reciprocally as

$$\frac{1}{r_1} = \frac{1}{k_{-1}} + \frac{k_{-1} + k_1 [\text{L}_1]}{k_{-1} k_2} \cdot \frac{1}{[\text{L}_2]}. \quad (10)$$

Eqn (10) shows that the rate parameter r_1 for the slowest transient will be hyperbolically dependent on $[\text{L}_2]$ for any fixed value of $[\text{L}_1]$, r_1 approaching k_{-1} at high concentrations of L_2 . If pre-equilibrations between enzyme and L_1 are carried out using ligand concentrations such that

$$\frac{k_2 [\text{L}_2]}{k_{-2}} \gg \frac{k_1 [\text{L}_1]}{k_{-1}} \gg 1. \quad (11)$$

Eqn (10) reduces to

$$\frac{1}{r_1} = \frac{1}{k_{-1}} \left(1 + \frac{k_1 [\text{L}_1]}{k_2 [\text{L}_2]} \right), \quad (12)$$

which agrees with the relationship previously derived for displacement reactions using a steady-state approximation [16]. When Eqn (12) applies, plots of $1/r_1$ vs $1/[\text{L}_2]$ will give straight lines with an intercept equal to $1/k_{-1}$ and a slope which provides direct information about the magnitude of the quotient k_1/k_2 .

The explicit expressions for amplitude coefficients A_{ij} in Eqn (6) are of little practical interest and will not be presented. It may suffice to state that the slower one of the two transients in Eqn (6) can be shown to

dominate ($A_{1i} > A_{2i}$) when the relationships in Eqn (5) or Eqn (11) are valid.

In the case when L_2 cannot be used at high enough concentration to fulfil Eqn (5), r_1 will vary non-hyperbolically with $[L_2]$. Estimates of k_{-1} may then be obtained by non-linear extrapolation to infinite concentration of L_2 using Eqn (9) as a regression function. Alternatively the apparent magnitude of k_{-2} can be decreased by carrying out reactions in the presence of ligands which bind rapidly, firmly, and specifically to the EL_2 species. The above theory will then apply to the transient concentration changes of EL_1 and the trapped EL_2 complex.

NAD^+ Dissociation Rates

Determinations of the rate constant for NAD^+ dissociation from liver alcohol dehydrogenase were performed between pH 6 and 10. Displacement of NAD^+ from the enzyme was effected by addition of varied concentrations of NADH, coenzymes being present in large excess to enzyme as well as to the respective equilibrium constant for coenzyme dissociation from the enzyme. To fulfil the requirements of Eqn (11) over the entire pH range tested, the apparent off-velocity constant for NADH was decreased by carrying out the reactions in the presence of 1 mM isobutyramide. This ligand is known to bind firmly and specifically to the enzyme · NADH complex, and control experiments established that the ligand concentration used was high enough to ensure a rapid equilibration with the enzyme · NADH complex. Under such conditions, application of the kinetic theory described in the preceding section is justified. Eqn (6) then predicts that the displacement process will be governed by two exponential transients, the rapid one of which (corresponding to the direct conversion of free enzyme into the trapped enzyme · NADH complex) can be anticipated to exhibit a low amplitude and a high rate ($r_2 \gg r_1$). According to Eqn (8) and previously reported data for coenzyme binding [2, 3, 8], the rapid transient would be expected to be too fast to be detected ($r_2 > 500 \text{ s}^{-1}$) when reactions are carried out at high NADH concentrations or at low pH.

This was confirmed experimentally. Reactions performed at pH values below 8.5 exhibited a single exponential transient at all NADH concentrations tested when monitored at 360 nm. Biphasic absorbance changes due to the displacement reaction were observed only with low NADH concentrations at high pH, and in such cases the initial rapid transient exhibited an amplitude at least 10-fold lower and a higher rate than the subsequent slow transient. Rate parameters r_1 for the (slow) exponential transient observed at each pH and fixed NAD^+ concentration were found to vary hyperbolically with the concen-

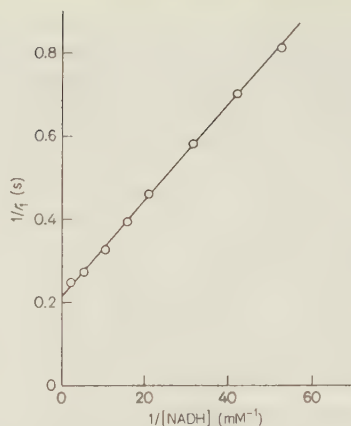


Fig.1. $NADH$ concentration dependence of the rate of displacement of NAD^+ from liver alcohol dehydrogenase. Enzyme (8 μM), pre-incubated with 0.6 mM NAD^+ , was reacted at 25 °C in 0.1 M ionic strength phosphate buffer (pH 9.3) with varied concentrations of NADH in the presence of 1 mM isobutyramide. Apparent first-order rate constants (r_1) for the displacement of NAD^+ were determined from the absorbance changes observed at 360 nm

tration of NADH. This is illustrated in Fig.1 by example of the results obtained at pH 9.3. As prescribed by Eqn (10), intercepts of the straight lines obtained in plots of $1/r_1$ vs $1/[NADH]$ (Fig.1) were taken to provide estimates of the reciprocal value of the rate constant for NAD^+ dissociation. This rate constant will be denoted k_{-1}' in accordance with the notation system introduced by Dalziel [4] for the reactions under consideration:



Fig.2 shows the variation with pH of the estimates of k_{-1}' thus obtained. Experimental data conform well to the generalized dissociation function

$$k_i = \frac{k_i^*}{1 + \frac{K_a}{[H^+]}} \quad (14)$$

where k_i^* denotes the limiting value of k_i at acid pH. The parameter estimates obtained on fitting Eqn (14) to the data in Fig.2 were $k_{-1}'^* = 160 (\pm 30) \text{ s}^{-1}$ and $pK_a = 7.6 (\pm 0.2)$.

$NADH$ Dissociation Rates

The rate of dissociation of NADH from liver alcohol dehydrogenase was studied by methods entirely analogous to those described above for NAD^+ dissociation. Rate parameters r_1 were determined at different pH between 6 and 10 for the (slow) exponen-

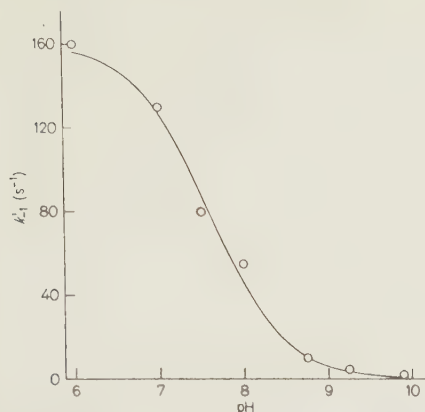


Fig. 2. Effect of pH on the NAD^+ dissociation rate constant (k_{-1}). Estimates of k_{-1} determined from the intercept of plots similar to and including the one in Fig. 1. The dissociation curve drawn was calculated from Eqn (14) for $k_{-1}^0 = 160 \text{ s}^{-1}$ and $\text{p}K_a = 7.6$.

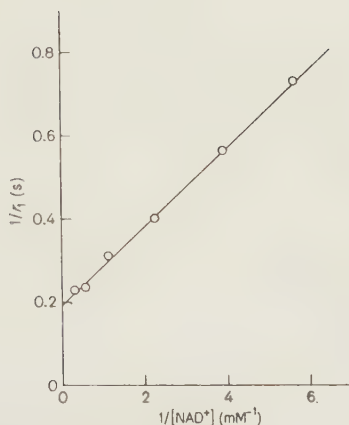


Fig. 3. NAD^+ concentration dependence of the rate of displacement of NADH from liver alcohol dehydrogenase. Enzyme ($5 \mu\text{M}$), pre-incubated with $50 \mu\text{M}$ NADH , was reacted at 25°C in 0.1 M ionic strength phosphate buffer ($\text{pH } 7.0$) with varied concentrations of NAD^+ in the presence of 5 mM pyrazole. Apparent first-order rate constants (r_1) for the displacement of NADH were determined from the absorbance changes observed at 297 nm .

Table 1. Effect of pH on the NADH dissociation rate constant (k_{-1}). Estimates of k_{-1} determined from the intercept of plots similar to and including that in Fig. 3.

pH	k_{-1}
	s^{-1}
6.0	$3.4 (\pm 0.4)$
7.0	$5.2 (\pm 0.6)$
8.0	$5.8 (\pm 0.4)$
8.8	$5.4 (\pm 0.5)$
9.3	$6.3 (\pm 0.8)$
10.0	$7.5 (\pm 1.0)$

tial transient observed on displacement of NADH from the enzyme by the addition of varied concentrations of NAD^+ . Fig. 3 illustrates that straight lines were obtained in plots of $1/r_1$ vs $1/[\text{NADH}^+]$ at each pH. Table 1 shows the estimates of the rate constant k_{-1} for NADH dissociation calculated from the intercept of such plots. The results in Table 1 confirm previous reports that there is no pronounced effect of pH on the NADH dissociation rate [4,8]. The magnitude of k_{-1} remains essentially constant at about 6 s^{-1} over the pH range investigated.

Coenzyme Association Rates

Pre-equilibration between enzyme and coenzyme in the above displacement experiments was performed under such conditions that application of Eqn (12) is justified. Consequently slopes of the straight lines obtained by the plotting procedure indicated in Fig. 1

and 3 provide information about the relative magnitude of on-velocity constants for the binding of NADH (k_1) and NAD^+ (k'_1). Fig. 4 shows the k_1/k'_1 ratios calculated from the slopes obtained at different pH for the displacement of NADH with NAD^+ and vice versa. The results in Fig. 4 provide direct evidence that the quotient k_1/k'_1 remains essentially constant between pH 6 and 10, indicating that on-velocity constants for coenzyme binding exhibit the same pH dependence over this pH range. In particular, the present results seem to exclude the possibility that k_1 and k'_1 are dependent on proton equilibria with distinct $\text{p}K_a$ of 9.0 and 10.0, respectively [9]. The pH profile for k_1/k'_1 expected in the latter case (dashed line in Fig. 4) is in obvious disagreement with the experimental observations.

Direct stop-flow kinetic studies of the effect of pH on coenzyme association rates were performed by more conventional methods, using a reporter ligand with well-characterized binding properties (*trans*-*N,N*-dimethylaminocinnamaldehyde [13]). Fig. 5 and 6 show the observed variation with pH of k_1 and k'_1 , respectively. The dissociation function in Eqn (12) can be well fitted to the experimental data using $k_1^* = 12 (\pm 2) \mu\text{M}^{-1} \text{ s}^{-1}$, $k_1'^* = 1.0 (\pm 0.2) \mu\text{M}^{-1} \text{ s}^{-1}$, and $\text{p}K_a = 9.2 (\pm 0.2)$.

Table 2 shows the equilibrium constants for coenzyme dissociation calculated from the rate constant estimates obtained in the present work. The kinetically determined equilibrium values agree satisfactorily with the binding constants obtained previously [2,3] from equilibrium measurements.

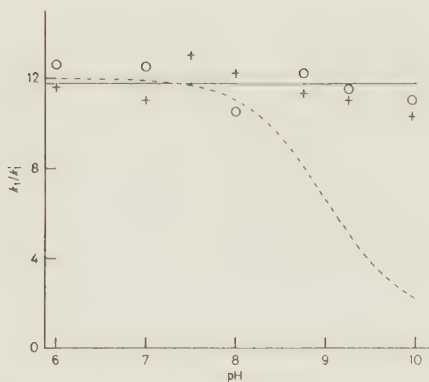


Fig. 4. Effect of pH on the relative magnitude of on-velocity constants for NADH (k_1) and NAD⁺ (k_1') binding to liver alcohol dehydrogenase. Estimates of the quotient k_1/k_1' obtained from the observed concentration dependence of the rate of NAD⁺ displacement with NADH (+) and vice versa (O). (---) pH profile calculated for k_1/k_1' with the assumption that k_1 and k_1' conform to Eqn (14) with $pK_a = 9.0$ and 10.0 respectively

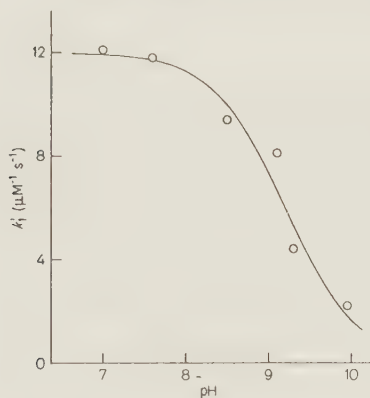


Fig. 5. Effect of pH on the NADH association rate constant (k_1). The dissociation curve drawn was calculated from Eqn (14) with $pK_a = 9.2$ and $k_1^* = 12 \mu\text{M}^{-1} \text{s}^{-1}$

Titrimetric Measurements

If the pK_a of an ionizing enzymic group is perturbed to a higher value on ligand binding to the enzyme, formation of the enzyme · ligand complex must result in a corresponding pH dependent uptake of protons from the reaction solution. Titrimetric experiments established that such a ligand-induced proton uptake occurs above pH 8 on the addition of saturating concentrations of NADH to a solution of liver alcohol dehydrogenase. As shown in Fig. 7, the amount of protons withdrawn from solution at different pH conforms well to a monobasic dissociation curve with a

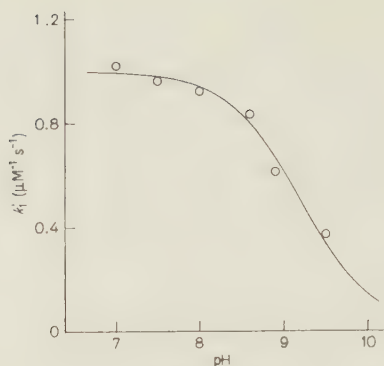


Fig. 6. Effect of pH on the NAD⁺ association rate constant (k_1'). The dissociation curve drawn was calculated from Eqn (14) with $pK_a = 9.2$ and $k_1^* = 1.0 \mu\text{M}^{-1} \text{s}^{-1}$

Table 2. Equilibrium constants for coenzyme dissociation from liver alcohol dehydrogenase

Estimates calculated from the present kinetic data compared to those obtained previously from equilibrium measurements. Rate constants are defined as in Eqn (13)

pH	k_{-1}/k_1 (NADH)		k'_{-1}/k'_1 (NAD ⁺)	
	reported [2]	calculated	reported [3]	calculated
	μM			
6	0.21	0.28	351	156
7	0.40	0.44	133	129
8	0.46	0.51	30.1	48
9	0.99	0.82	9.3	10
10	5.0	4.6	5.5	4.6

pK_a agreeing with that (9.2) controlling the rate of coenzyme association to the enzyme. The stoichiometry of proton uptake approaches one proton per active site of the enzyme at high pH. The addition of saturating concentrations of AMP or $\text{Pt}(\text{CN})_4^{2-}$ [17] to the enzyme resulted in a similar uptake of approximately half an equivalent of protons at pH 9.2, whereas no proton uptake or release occurred on formation of the binary enzyme · adenosine complex.

The pK_a regulating NAD⁺ association to free enzyme was similarly checked by measurement of the stoichiometry of proton release due to NAD⁺ binding. To avoid interferences from the pK_a 7.6 group which regulates NAD⁺ dissociation, reactions were performed in the presence of 1 mM trifluoroethanol; this ligand is known to perturb the pK_a of the latter group to a value below 5 [10]. The results confirmed that ternary complex formation between enzyme, NAD⁺, and trifluoroethanol leads to the

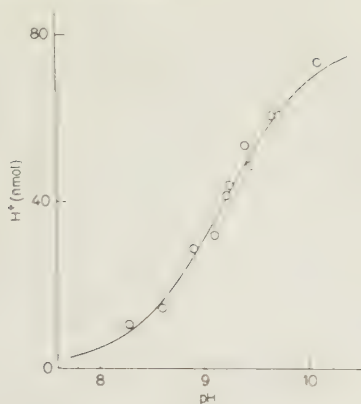


Fig. 7. Effect of pH on proton uptake resulting from the binding of $NADH$ to liver alcohol dehydrogenase. Titrimetrically determined amount of protons withdrawn from solution on reacting the enzyme (80 nmol) with $NADH$ (0.5 mM) in 33 mM sodium sulphate at 25 °C. The curve drawn corresponds to a monobasic dissociation function with a pK_a of 9.2

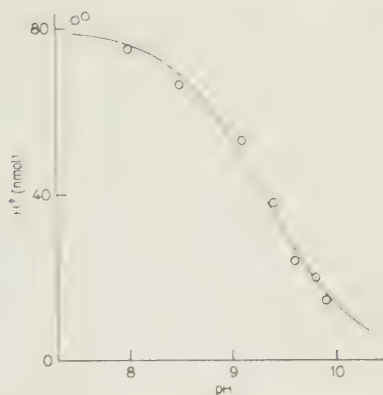


Fig. 8. Effect of pH on proton release resulting from the binding of NAD^+ and trifluoroethanol to liver alcohol dehydrogenase. Titrimetrically determined amount of protons released on reacting the enzyme (80 nmol) with NAD^+ (200 μ M) and trifluoroethanol (1 mM) in 33 mM sodium sulphate at 25 °C. The curve drawn corresponds to a monobasic dissociation function with a pK_a of 9.3

release of approximately one proton per active site below pH 8 [10]. At higher pH the amount of protons liberated decreases towards zero, half an equivalent of protons being released at pH 9.3 (Fig. 8).

DISCUSSION

Ligand displacement experiments have been previously used for the determination of ligand dissociation rates in enzymic reaction systems. The present theoretical treatment of the transient-state kinetics inherent in Eqn (1) provides analytical relationships for the evaluation and interpretation of such experiments. A main advantage with these relationships is that they establish the functional dependence on ligand concentrations of the observed displacement rates. This makes it possible to obtain accurate estimates of the off-velocity constant for the displaced ligand by extrapolation procedures based on regression functions of an adequate form. Under the conditions of typical displacement experiments, extrapolation to infinite concentration of the displacing ligand can be reliably performed in terms of a hyperbolic relationship [Eqn (10)].

Extrapolation procedures were not applied by DeTraglia et al. in their determination of NAD^+ dissociation rates in the liver alcohol dehydrogenase system; displacement rates (r_1) observed at different pH values for the chosen fixed combination of co-enzyme concentrations were assumed to provide a direct measure of the rate constant for NAD^+ dissociation [8]. Calculations based on Eqn (10) for the ligand concentrations and rate constant estimates

given by DeTraglia et al. (or the estimates given by us) show that this assumption is dubious owing to the use of an insufficiently high concentration of $NADH$ for displacement of NAD^+ from the enzyme. The assumption of DeTraglia et al. leads them to underestimate the magnitude of the rate constant (k'_{-1}) for NAD^+ dissociation by a factor varying from about 1.5 at high pH to about 3 at low pH. This explains why their reported k'_{-1} values are unrealistically low (significantly lower than the turnover rate of enzymic aldehyde reduction [4]), fit badly to a monobasic dissociation function [8] and lead to an overestimation of the pK_a value regulating the rate of NAD^+ dissociation. When adequately interpreted in view of Eqn (10), the data of DeTraglia et al. seem to be entirely consistent with the present conclusion that NAD^+ dissociation exhibits a pK_a 7.6 dependence. The present results, therefore, lend strong support to the suggestion of Shore et al. [10] that substrate binding to, and NAD^+ dissociation from, the enzyme $\cdot NAD^+$ complex are controlled by the same ionizing group with a pK_a of 7.6.

A second advantage with the analytical relationships now derived for displacement reactions is that they can be used for interpretation of the ligand concentration dependence of the observed displacement rates. As indicated by Eqn (12), for example, reactions may be carried out under such conditions that the kinetic data provide estimates of the quotient between on-velocity constants for the two competing ligands. This makes it possible to design and perform sensitive experimental tests aimed directly at a detection of differences in the pH dependence of the on-velocity constants. The present application of such critical

tests to the liver alcohol dehydrogenase system (Fig. 4) provides evidence in support of the report of DeTraglia et al. [8] that there is no significant difference in the pH dependence of association rates for NADH and NAD^+ over the pH range examined. This result, the kinetic data in Fig. 5 and 6, and the titrimetric data in Fig. 7 and 8 lead us to conclude that on-velocity constants for coenzyme binding are dependent on one and the same proton equilibrium exhibiting a pK_a close to 9.2. The latter estimate of the pK_a regulating coenzyme association is slightly lower than that (9.5) obtained by DeTraglia et al. and agrees more closely with the pK_a value (9.0) reported for NADH association by Shore et al. [9].

In a previous report from this laboratory it was suggested that the decreased stability of the enzyme $\cdot$ NADH complex above pH 9 reflects an ionization process which affects the anion-binding capacity of the coenzyme-binding site [17]. This suggestion was based on evidence indicating that an ionizing group with a pK_a close to 9 regulates the binding of NADH, coenzyme-competitive anions such as $\text{Pt}(\text{CN})_2^{2-}$, and coenzyme fragments such as ADP-ribose and AMP, whereas there is no similar effect of pH on the binding of adenosine. The present investigation lends strong support to this idea in showing that NADH, AMP, and $\text{Pt}(\text{CN})_2^{2-}$, but not adenosine, induce proton uptake corresponding to a pK_a of 9.2 on binary-complex formation with the enzyme. This observation provides evidence that the former three ligands bind preferentially to the enzyme form in which the pK_a 9.2 group is in the protonated state, the pK_a of the group being perturbed to a value above 10 in the binary complexes.

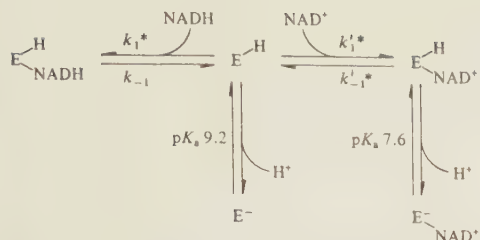
The present observation that NAD^+ association exhibits the same pH dependence as NADH association provides additional evidence that ionization of the pK_a 9.2 group has a general effect on complex formation with ligands which utilize the anion-binding part of the coenzyme-binding site. It should be noted, however, that this effect not necessarily is caused by (not even is likely to be caused by) ionization of one of the groups which form part of the coenzyme-binding site. This follows from the fact that ionization of the pK_a 9.2 group affects only the on-velocity constant for coenzyme binding. The latter observation might indicate that the pK_a 9.2 ionization step converts the enzyme into a conformational state in which the anion-binding region of the coenzyme-binding site has been sterically blocked or otherwise modified to render the enzyme unable to associate properly with the ligands. Such a conformational change, in principle, could be triggered by any ionizing group in the enzyme.

In particular it cannot be excluded that the conformational change may be triggered by ionization of the same group as the one giving rise to the pK_a 7.6 dependence of the NAD^+ dissociation rate. To be consistent

with the present kinetic data, this would require that the pK_a of the ionizing group is 9.2 in free enzyme, decreasing to 7.6 on the binding of NAD^+ and increasing to a value above 10 on the binding of NADH. The idea that the two pK_a values regulating NAD^+ binding can be attributed to a single ionizing group was introduced by Dalziel in his pH dependence study of the steady-state kinetics of liver alcohol dehydrogenase catalysis [4]. The NAD^+ -induced shift in pK_a of the ionizing group was explained as an effect of electrostatic interaction, and the group was tentatively identified as a water molecule bound to the catalytic zinc atom of the enzyme. These ideas have been accepted in several later reports [1], and recent crystallographic results led to the suggestion that zinc-bound water, hydrogen bonded to a Ser-48/His-51 proton relay system, represents the actual ionizing structure giving rise to the pK_a 7.6 and 9.2 dependences of NAD^+ binding [18].

To our opinion the absence of a pronounced effect of pH on the rate of NADH dissociation may be taken as direct evidence that the pK_a 7.6 dependence of NAD^+ dissociation derives from an electrostatically perturbed group. The present and previously reported data for NAD^+ binding [1] consistently indicate that the pK_a of this group is shifted by at least 1.4 units on binary-complex formation with the positively charged coenzyme. Such a large electrostatic pK_a perturbation can occur only with groups located in proximity to the nicotinamide ring of the enzyme-bound NAD^+ . For these reasons, and in view of the structural information now available for the enzyme [1], we agree that the pK_a 7.6 dependence of NAD^+ dissociation most likely reflects the pK_a of zinc-bound water (possibly linked to the Ser-48/His-51 proton relay system) in the binary enzyme $\cdot$ NAD^+ complex.

It should be emphasised, however, that no information is available about the pK_a of zinc-bound water in the absence of NAD^+ . In particular, no evidence has been presented to indicate that the pK_a of zinc-bound water is actually as low as 9.2 in the free enzyme. A higher pK_a seems quite reasonable in view of the fact that the catalytic zinc atom is coordinated to two negatively charged cysteinyl residues in the protein [1]. Since the nicotinamide part of the coenzyme is known to be bound near the zinc atom in a hydrophobic environment [1], there would be no difficulty to account for a NAD^+ -induced electrostatic perturbation of the pK_a of zinc-bound water by considerably more than 1.4 units. It might be more difficult to explain a shift in the pK_a of this group from an assumed value of 9.2 in free enzyme to a value above 10 in the binary enzyme $\cdot$ NADH complex. For these reasons, the possibility cannot be excluded that the pK_a of zinc-bound water is higher than 10 in free enzyme and in the enzyme $\cdot$ NADH complex, decreasing to 7.6 on the binding of NAD^+ . This would



Scheme 1. Mechanism accounting for the pH dependence of coenzyme binding to liver alcohol dehydrogenase

imply that the pK_a 9.2 dependence of coenzyme association derives from a second ionizing group, the identity of which remains unknown. The binding mechanism shown in Scheme 1 accounts for the present experimental results without giving any indication as to the identity of the pK_a 7.6 and 9.2 group(s).

From a descriptive point of view, the present investigation of coenzyme binding to liver alcohol dehydrogenase resolves the two major ambiguities mentioned in the introduction. Only two proton equilibria (pK_a 7.6 and 9.2) have to be considered to account for the main effects of pH on coenzyme binding between pH 6 and 10, and one of these equilibria (pK_a 7.6) can be assumed to be identical with that reported to affect the binding of inhibitory substrate analogues to the enzyme $\cdot NAD^+$ complex [10]. It cannot be decided from the present kinetic data whether the two proton equilibria are attributable to two distinct ionizing groups on the enzyme, or to a single group exhibiting distinct pK_a in different enzyme species. Irrespectively of the identity of the ionizing group(s) involved, however, Scheme 1 provides an adequate formal description of the observed pH dependence of coenzyme binding to the enzyme. In a future publication we will show how the binding mechanism proposed in Scheme 1 can be reconciled

with the observed effect of pH on other reaction steps in the catalytic mechanism.

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Effect of pH on the Process of Ternary-Complex Interconversion in the Liver-Alcohol-Dehydrogenase Reaction

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1. Kinetic relationships referring to multiple-turnover conditions have been derived for the slowest exponential transient appearing in two-substrate enzyme reactions proceeding by an ordered ternary-complex mechanism. The validity of these and previously derived theoretical relationships for this mechanism has been tested by application to the liver alcohol dehydrogenase reaction.

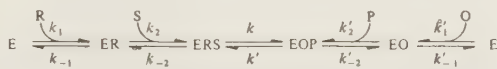
2. All essential features of the transient-state kinetics of alcohol oxidation by NAD^+ in the liver alcohol dehydrogenase system can be qualitatively and quantitatively explained in view of the compulsory-order mechanism in the proposed scheme. There is no kinetic evidence for any half-of-the-sites reactivity of the enzyme. A consistent set of rate constants is reported for the enzymic oxidation of benzyl alcohol at pH 8.75.

3. Transient-state rate parameters for benzyl alcohol/benzaldehyde catalysis by liver alcohol dehydrogenase have been determined at different pH. The interpretation of such rate parameters is critically discussed with reference to their informative value for the purpose of determination of rate constants (k and k') for the process of ternary-complex interconversion in the proposed scheme. It is concluded that the apparent rate constant (k') for hydride transfer from benzyl alcohol to NAD^+ is dependent on a proton dissociation step with a pK_a of 6.4, whereas the rate constant (k) for hydride transfer from NADH to benzaldehyde exhibits no corresponding dependence on proton association.

4. The asymmetric pH dependence of the forward and reverse rate of ternary-complex interconversion during liver alcohol dehydrogenase catalysis appears to reflect an obligatory step of alcohol/alcoholate ion equilibration occurring at the ternary-complex level. It is suggested that the observed pK_a 6.4 dependence of the transient rate of alcohol oxidation can be attributed to a coupled acid-base system involving minimally the enzyme-bound alcohol and the protein residues Ser-48 and His-51.

Liver alcohol dehydrogenase, which catalyzes the reversible oxidation of various alcohols by NAD^+ , has been extensively investigated [1]. The enzyme is known to operate by an effectively ordered ternary-complex mechanism (Scheme 1), with coenzyme-binding preceding the binding of substrate. The stoichiometry of the overall reaction prescribes that a proton is liberated for every NADH molecule formed, which indicates that the catalytic reaction is critically dependent on one or several proton transfer steps. Such proton transfers would be expected to involve ionizing groups at the active center of the enzyme; unmediated proton transfer from the catalytic reaction center to the solution is rendered unlikely by crystallographic data [1].

Evidence for the mechanistic importance of ionizing groups at the active center of liver alcohol dehydrogenase has been obtained by examination of the pH dependence of individual reaction steps in the catalytic mechanism [1]. Such studies, however, have led to different conclusions as concerns the effect of pH on the hydride transfer process (the steps of ternary-complex interconversion in Scheme 1). Brooks et al. reported that the rate of hydride transfer from



Scheme 1. The ordered ternary-complex mechanism considered in the present investigation. E, R, and O denote enzyme, NADH, and NAD^+ , respectively. S stands for an aldehyde substrate and P for the corresponding alcohol

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

ethanol to NAD^+ , as determined by transient-state kinetic techniques under multiple-turnover conditions, is dependent on a dissociating group with a pK_a of 6.4 [2]. McFarland et al. later carried out similar experiments under single-turnover conditions, from which they concluded that ternary-complex interconversion cannot be subject to acid-base catalysis by a protonic acid with a pK_a between 6 and 10 [3]. The latter authors found that the rate of hydride transfer from NADH to aromatic aldehydes is nearly independent of pH within the range 6–10, and they could not detect any pK_a -6.4 dependence of the transient rate of enzymic benzyl alcohol oxidation by NAD^+ .

The present investigation was undertaken in order to resolve the above controversy. Particular attention has been paid to possible ambiguities in the interpretation of the transient-state kinetic parameters examined by Brooks et al. and by McFarland et al. [2, 3]. One of us has previously derived an analytical solution for the transient-state kinetics inherent in Scheme 1, applicable to data obtained under single-turnover conditions [4]. In the present paper we consider the solution corresponding to multiple-turnover conditions, and the effect of enzyme cycling on the transient reaction is examined and discussed. It is shown that transient-state kinetic data for the enzymic oxidation of benzyl alcohol by NAD^+ can be quantitatively explained in terms of equivalent and independent catalytic sites, and the informative value of such data for the purpose of determination of rate constants in Scheme 1 is critically analyzed. Finally, data are reported indicating that the transient rate of enzymic benzyl alcohol oxidation exhibits a pH dependence similar to that observed for the enzymic oxidation of ethanol [2]. A mechanism is proposed which accounts for the observations made.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse-liver alcohol dehydrogenase from Boehringer Mannheim GmbH was further purified as described by Bernhard et al. [5]. Enzyme concentrations were determined spectrofluorimetrically by titration with NADH in the presence of isobutyramide [6], and are reported throughout as active-site concentrations.

The coenzymes NADH and NAD^+ were purchased from Sigma Chemical Corp. (grade III) and were used without further purification. $[4\text{-}^2\text{H}]\text{NADH}$ was prepared by the method of Rafter and Colowick according to the description of McFarland et al. [7]. Substrates and other reagents used were of analytical grade. Benzyl alcohol and benzaldehyde were vacuum-distilled under nitrogen prior to use.

Buffer solutions were prepared using water twice distilled from a quartz apparatus. Experiments at pH 8.75 were performed in 0.05 M pyrophosphate buffer. Kinetic pH dependence experiments were carried out in buffers of ionic strength 0.1 M using bicarbonate at pH values above 9, phosphate at pH 6 through 8.5, and phosphate/acetate at pH values below 6. The pH of reaction solutions was routinely measured both before and after each experiment and pH values stated may be considered to be accurate within 0.02 unit.

Kinetic Measurements

All experiments reported in the present investigation were performed at 25 °C. Steady-state kinetic parameters for the enzymic oxidation of benzyl alcohol by NAD^+ were determined spectrophotometrically as described previously [8]. Transient-state kinetic measurements were made spectrophotometrically by stopped-flow techniques. The dissociation rate of NADH at pH 8.75 was determined by reacting the binary enzyme·NADH complex with NAD^+ in the presence of pyrazole [9]. Enzyme (16 μM) preincubated with NADH (100 μM) in one syringe was mixed with NAD^+ (0.1–1.8 mM) and pyrazole (10 mM) from the second syringe, reactions being monitored at 297 nm.

The transient-state kinetics of enzymic benzyl alcohol oxidation by NAD^+ were examined at 328 nm (the isosbestic point for free and enzyme-bound NADH [10]) by mixing substrate (0.04–40 mM), preincubated with coenzyme (usually 2 mM) in one syringe, with enzyme (10–14 μM) from the second syringe. All reactants were in the same buffer solution, adjusted to the same pH. When required to obtain single-turnover conditions, 200 mM isobutyramide was added to the solution containing substrate and coenzyme [11]. The amount of NADH produced during catalysis at pH 8.75 was calculated using a difference absorption coefficient of 5400 (4750) $\text{M}^{-1}\text{cm}^{-1}$ for reactions carried out in the absence (presence) of isobutyramide [11]. Rate determinations at pH values below 6 were performed by a slightly modified procedure to prevent partial denaturation of the enzyme prior to reaction. The enzyme was kept at pH 6.0 in a 0.04 M phosphate buffer solution (containing 17 mM Na_2SO_4) prior to mixing, while reactants in the second syringe were in 0.05 M acetate buffer adjusted to a lower pH. The pH of the reaction solution after mixing was estimated on the basis of pilot experiments, and was checked by direct measurement after completed reaction. Rate parameters calculated from the pH dependence experiments were determined by extrapolation to infinite substrate concentration and are thus not affected by the presence of buffer ions (acetate) competitive with the alcohol substrate.

Transient-state rate parameters for the enzymic reduction of benzaldehyde by $[4\text{-}^2\text{H}]\text{NADH}$ were determined at 328 nm in the presence of pyrazole, as described for the corresponding reaction involving NADH [12].

The above data refer to concentrations of solutions before mixing in the stopped-flow apparatus. Values stated in the following sections refer to concentrations after mixing, i.e. at the start of the actual reaction.

Rapid-Reaction Equipment

Transient-state kinetic experiments were carried out with a Durum D-130 stopped-flow photometer combined to a Beckman DU monochromator. Photomultiplier signals were visualized in a Tektronix 5103N storage oscilloscope, and were registered and digitalized in a Datalab DL 905 transient recorder. Apparently single-exponential transients were digitalized at 1024 equidistant sample points, distributed over a period of time covering at least 95% of the observed absorbance changes. For the examination of biphasic processes (two exponential transients, or one transient superimposed on steady-state absorbance changes) sampling was performed over two different time scales, 400–600 equidistant sample points being registered within each phase. Registered data were (after analogue conversion) written out on a Hewlett-Packard 7005B XY recorder, or were read into a CompuCorp 425G computer for statistical evaluation.

Data Processing

Transient-state kinetic data were analyzed in view of the regression function in Eqn (1), which describes the dependence of the observed absorbance changes ΔA on time t in terms of a steady-state contribution (αt) combined to two exponential transients with amplitudes β_j and rate parameters r_j , $j = 1, 2$ [13].

$$\Delta A = \alpha \cdot t + \sum_{j=1}^n \beta_j (e^{-r_j t} - 1). \quad (1)$$

Preliminary estimates of the kinetic parameters in Eqn (1) were determined by standard graphical procedures. Final parameter estimates were calculated statistically, using previously described non-linear regression methods [12]. In cases where contributions from a second exponential transient were found to be statistically insignificant, the single-exponential regression function obtained by putting $n = 1$ in Eqn (1) was fitted to the experimental observations. Similarly, α was put equal to zero on fitting Eqn (1) to experimental data exhibiting no significant contributions from steady-state absorbance changes.

When required for accurate determination of amplitudes β_j , the experimental time scale defined

by the stop of the flow was corrected for a dead-time of 3 ms of the stopped-flow apparatus, determined as described by Gutfreund [14]. Transient-state kinetic parameter estimates plotted in Fig. 1–3 represent means of 2–4 separate determinations.

RESULTS

Transient-State Kinetic Relationships for Scheme 1 under Cycling Conditions

The kinetic differential equations for Scheme 1 can be analytically solved with the assumption that pseudo first-order conditions prevail [4]. The solution obtained prescribes that the pre-steady-state kinetics of Scheme 1 are governed by four exponential transients which, in the case of cycling systems, are superimposed on the steady-state changes of the concentration variable(s) examined [13]. The transient-state kinetics of reactions catalyzed by liver alcohol dehydrogenase can be conveniently studied under pseudo-first-order conditions (obtained, for instance, by using substrate and coenzyme in large excess to enzyme) by monitoring changes in the total concentration of NADH [12]. Under such conditions, two of the four exponential transients corresponding to Scheme 1 may be of kinetic significance when the reaction is carried out in the direction of aldehyde reduction, whereas the reverse reaction of alcohol oxidation would be expected to be governed by a single kinetically significant transient (the slowest one) [4, 12]. For this reason, the present theoretical analysis will be restricted to an examination of the effect of enzyme cycling on the slowest exponential transient corresponding to Scheme 1.

According to Scheme 1, the following kinetic differential equations apply to the enzyme(E)-catalyzed oxidation of an alcohol (P) by NAD^+ (O) in the initial absence of NADH (R) and the aldehyde product (S):

$$\begin{aligned} \frac{d[\text{EO}]}{dt} &= k'_1 c_0 \\ &\quad (c_E - [\text{EO}] - [\text{EOP}] - [\text{ERS}] - [\text{ER}]) \\ &\quad - (k'_{-1} + k'_2 c_P) [\text{EO}] + k'_{-2} [\text{EOP}] \end{aligned} \quad (2)$$

$$\begin{aligned} \frac{d[\text{EOP}]}{dt} &= k'_2 c_P [\text{EO}] - (k' + k'_{-2}) [\text{EOP}] \\ &\quad + k [\text{ERS}] \end{aligned} \quad (3)$$

$$\frac{d[\text{ERS}]}{dt} = k' [\text{EOP}] - (k + k_{-2}) [\text{ERS}] \quad (4)$$

$$\frac{d[\text{ER}]}{dt} = k_{-2} [\text{ERS}] - k_{-1} [\text{ER}]. \quad (5)$$

It is assumed that initial concentrations c_E , c_O , and c_P of the respective reactants are such that $c_E \ll c_O$, c_P . The secular equation for Eqns (2–5) can be expressed as

$$\begin{vmatrix} r' - (k'_{-1} + k'_1 c_O + k'_2 c_P) & k'_{-2} - k'_1 c_O \\ k'_2 c_P & r' - (k'_{-2} + k') \\ 0 & k' \\ 0 & 0 \\ -k'_1 c_O & -k'_1 c_O \\ k & 0 \\ r' - (k + k_{-2}) & 0 \\ k_{-2} & r' - k_{-1} \end{vmatrix} = 0.$$

Development of this determinant gives a fourth-degree equation

$$r'^4 - p_3 r'^3 + p_2 r'^2 - p_1 r' + p_0 = 0 \quad (6)$$

in which

$$p_0 = k_{-1} k'_2 k' k_{-2} [k'_1 c_O (\Phi'_0 c_P + \Phi'_2) + c_P + k'_{-1} \Phi'_2] \quad (7)$$

$$\begin{aligned} p_1 = & k'_1 c_O [k'_2 k' k_{-2} \Phi'_2 \\ & + k_{-1} (k + k' + k_{-2} + k'_{-2}) \\ & + k'_2 c_P (k + k' + k_{-2} + k_{-1}) \\ & + k_{-1} [k'_2 k' k_{-2} \Phi'_2 \\ & + k'_{-1} (k + k' + k_{-2} + k'_{-2}) \\ & + k'_2 k' k_{-2} (k_{-1} \Phi'_0 c_P + k'_{-1} \Phi'_2)]. \end{aligned} \quad (8)$$

The parameters Φ'_2 and Φ'_0 stand for the steady-state kinetic Dalziel coefficients [15] for the enzymic reaction and are given by

$$\Phi'_2 = \frac{k' k_{-2} + k'_{-2} (k + k_{-2})}{k'_2 k' k_{-2}} \quad (9)$$

$$\Phi'_0 = \frac{1}{r'_{1,\infty}} + \frac{1}{k_{-1}} \quad (10)$$

$$r'_{1,\infty} = \frac{k' k_{-2}}{k + k' + k_{-2}} \quad (11)$$

The four roots of Eqn (6) represent the rate parameters of the four exponential transients governing the pre-steady-state kinetics of the reaction [4]. Assuming that one of the roots is considerably smaller than the remaining three roots ($r'_{1c} \ll r'_{jc}, j = 2, 3, 4$; subscript c stands for cycling conditions), the rate parameter r'_{1c} of the slowest transient is given approximately [4] by

$$r'_{1c} = \frac{p_0}{p_1} \quad (12)$$

Eqns (7–12) provide an analytical expression for the dependence of r'_{1c} on the concentrations of sub-

strate and coenzyme. At saturating concentrations of coenzyme this expression reduces to

$$r'_{1c} = \frac{k_{-1} k'_2 k' k_{-2}}{k'_2 (k + k' + k_{-2} + k_{-1}) c_P + k'_2 k' k_{-2} (\Phi'_0 c_P + \Phi'_2)} \quad (13)$$

This should be compared to the corresponding relationship referring to single-turnover conditions ($k_{-1} = 0$), which may be written reciprocally as [4]

$$\frac{1}{r'_{1c}} = \frac{1}{r'_{1,\infty}} + \frac{\Phi'_2}{c_P} \quad (14)$$

Eqn (13) cannot be similarly linearized. Insertion of Eqns (10–11) into Eqn (13) shows that r'_{1c} changes non-hyperbolically with increasing substrate concentration towards a limiting value $r'_{1c,\infty}$ given by

$$r'_{1c,\infty} = \frac{k' k_{-2} + k_{-1} (k + k' + k_{-2})}{k + k' + k_{-2} + k_{-1}} \quad (15)$$

When $k' k_{-2} \gg (k_{-1})^2$, Eqn (15) reduces to

$$r'_{1c,\infty} = k_{-1} + r'_{1,\infty} \quad (16)$$

A relationship for the amplitude (A'_{1c}) of the slowest exponential transient can be similarly derived [4] with the assumption that $r'_{1c} \ll r'_{jc}, j = 2, 3, 4$. This relationship is given by

$$A'_{1c} = v' \left(\frac{1}{r'_{1c}} - \frac{1}{k_{-2}} \right) \left(\frac{r'_{1c}}{k_{-1}} - 1 \right) \quad (17)$$

where v' denotes the steady-state reaction velocity. At saturating concentrations of substrate and coenzyme Eqn (17) reduces to

$$A'_{1c,\infty} = \frac{c_E}{\Phi'_0} \left(\frac{1}{r'_{1c,\infty}} - \frac{1}{k_{-2}} \right) \left(\frac{r'_{1c,\infty}}{k_{-1}} - 1 \right) \quad (18)$$

When $k_{-2} \gg k \gg k', k_{-1}$ Eqn (16) is valid and Eqn (11) reduces to $r'_{1,\infty} = k'$. Under such conditions it follows from Eqns (10) and (18) that

$$A'_{1c,\infty} = \frac{c_E}{\left(1 + \frac{k_{-1}}{k'} \right)^2} \quad (19)$$

Steady-State Kinetic Experiments

Steady-state kinetic Dalziel coefficients for the liver-alcohol-dehydrogenase-catalyzed oxidation of benzyl alcohol by NAD^+ were determined by standard spectrofluorimetric techniques. The parameter estimates obtained at pH 8.75 were $\Phi'_0 = 0.26 (\pm 0.03) \text{ s}$

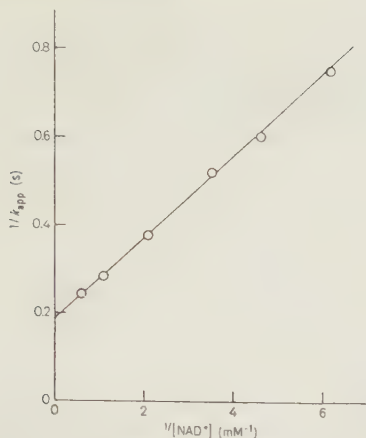


Fig. 1. Rate of dissociation of NADH from the binary enzyme · NADH complex. Apparent first-order rate constants (k_{app}) for the displacement process observed at 297 nm on reacting liver alcohol dehydrogenase (8 μM), pre-incubated with NADH (50 μM), with varied concentrations of NAD^+ (0.15–1.8 mM) at 25 °C in 0.05 M pyrophosphate buffer (pH 8.75) containing 5 mM pyrazole

and $\Phi_2' = 7.4 (\pm 0.6) \text{ s} \cdot \mu\text{M}$. These estimates agree with the steady-state rate parameter values reported by McFarland et al. [3].

Determination of the Rate Constant for NADH Dissociation

The magnitude at pH 8.75 of the rate constant k_{-1} in Scheme 1 was determined by displacement of NADH from the binary enzyme · NADH complex using NAD^+ in the presence of 5 mM pyrazole. Apparent first-order rate constants (k_{app}) for the displacement process were determined as a function of the NAD^+ concentration. As indicated by the double-reciprocal plot in Fig. 1, k_{app} was found to increase hyperbolically with increasing concentrations of NAD^+ towards a maximum value of $5.4 (\pm 0.5) \text{ s}^{-1}$. The latter value may be taken as an estimate of k_{-1} at pH 8.75.

Transient-State Kinetics of Benzyl Alcohol Oxidation

The transient-state kinetics of the enzymic oxidation of benzyl alcohol by NAD^+ were examined by stopped-flow techniques at 328 nm. Reactions were carried out at pH 8.75, substrate and coenzyme being used in large excess to enzyme to justify evaluation of the kinetic data in terms of exponential transients. Varied concentrations of benzyl alcohol (0.02–20 mM) were reacted with a fixed high concentration

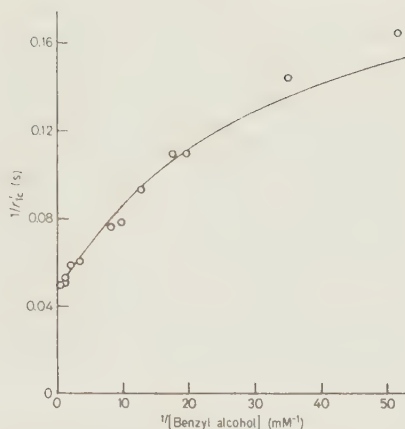


Fig. 2. Concentration dependence of the transient rate of enzymic benzyl alcohol oxidation under cycling conditions. Rate parameters (r'_e) for the exponential burst of NADH production observed at 328 nm on reacting 1 mM NAD^+ with varied concentrations of benzyl alcohol (0.02–2 mM) at 25 °C in 0.05 M pyrophosphate buffer (pH 8.75) containing about 6 μM liver alcohol dehydrogenase. The curve drawn was calculated from Eqn (13) using the data given in Table 2

of NAD^+ (usually 1 mM) in the presence of about 5 μM enzyme. Increasing the NAD^+ concentration from 1 to 5 mM had no significant effect on the rate of the exponential transient observed, indicating that 1 mM NAD^+ may be considered as a saturating concentration of coenzyme in this respect. Reactions were performed under cycling conditions, as well as under single-turnover conditions. In the latter case, reaction solutions contained 100 mM isobutyramide to prevent cycling of the enzyme [3, 11].

The results obtained agreed well with the observations made in previous transient-state kinetic studies of the same reaction [3, 11, 16, 17] and may be briefly summarized as follows. Under cycling conditions, there is a single-exponential burst of NADH formation preceding the steady-state phase of coenzyme reduction. The amplitude of the burst was found to tend towards a limiting value corresponding to $0.51 (\pm 0.04)$ mol of NADH produced per active site of enzyme when the concentration of substrate was raised from 20 μM to 1 mM. At substrate concentrations above 1 mM, where substrate inhibition of the steady-state reaction velocity was observed, the burst amplitude approached the active site concentration of enzyme. The dependence on substrate concentration of apparent first-order rate constants r'_e for the exponential burst is shown in Fig. 2. The magnitude of r'_e increases with increasing non-inhibitory substrate concentrations towards a limiting value of $21 (\pm 2) \text{ s}^{-1}$, which may be taken as an estimate of the parameter $r'_{e, \infty}$. At

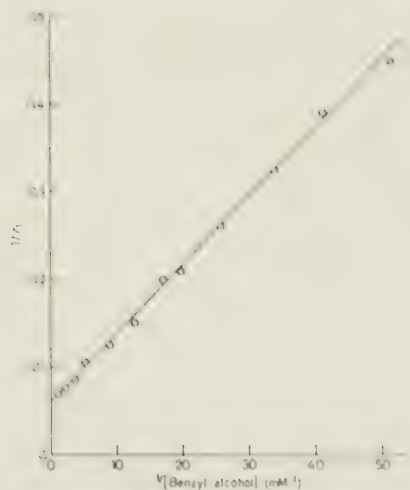


Fig. 3 Concentration dependence of the transient rate of enzymic benzyl alcohol oxidation under single-turnover conditions. Conditions as in Fig. 2, except that reactions were carried out in the presence of 100 mM isobutyramide

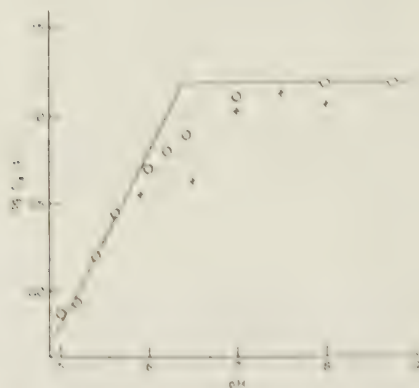


Fig. 4 pH dependence of the transient rate of enzymic benzyl alcohol oxidation. Maximum values ($r'_{i,\infty}$) of rate parameters r_i for the transient rate of benzyl alcohol oxidation under single-turnover conditions were determined by extrapolation to infinite substrate concentration as indicated in Fig. 3. Data reported by McFarland et al. [3] are included (+) for comparison

inhibitory benzyl alcohol concentrations, the apparent first-order rate constant for the transient was found to decrease slightly in magnitude towards that observed under single-turnover conditions.

The presence of 100 mM isobutyramide in the reaction solution caused an almost complete depression of the steady-state reaction velocity; NADH production was restricted essentially to a single-exponential process with an amplitude corresponding to about 95% of the active site concentration of enzyme. The variation with substrate concentration of the rate parameter r'_i for this exponential transient is shown in Fig. 3. In consistence with Eqn (14), $1/r'_i$ exhibits a linear dependence on the reciprocal concentration of substrate. An adequately weighted least-squares fit of Eqn (14) to the experimental data gave $r'_{i,\infty} = 16 (\pm 2) \text{ s}^{-1}$ and $\Phi'_i = 7.8 (\pm 1.2) \text{ s} \cdot \mu\text{M}$ (corresponding to the intercept and slope, respectively, of the straight line in Fig. 3). The latter value agrees well with the estimate of Φ'_i ($7.4 \text{ s} \cdot \mu\text{M}$) obtained from the steady-state kinetic experiments.

Effect of pH on the Transient Rate of Benzyl Alcohol Oxidation

The transient-state rate parameter $r'_{i,\infty}$ for the enzymic oxidation of benzyl alcohol by NAD^+ was determined at different pH between 5 and 9 as described above for the reaction at pH 8.75. The results are shown in Fig. 4, from which it can be seen that

the magnitude of $r'_{i,\infty}$ decreases most significantly below pH 7. Regression analysis of the data in Fig. 4 established that the variation of $r'_{i,\infty}$ with pH can be described in terms of a dependence on a proton dissociation reaction exhibiting an apparent pK_a of $6.4 (\pm 0.1)$. The estimate obtained for the limiting value of $r'_{i,\infty}$ at high pH was $16 (\pm 2) \text{ s}^{-1}$.

Effect of pH on the Transient Rate of Benzaldehyde Reduction

The enzymic reduction of benzaldehyde by NADH, monitored at 328 nm under single-turnover conditions, is known to be governed by two kinetically significant exponential transients [12]. The maximum value $r_{2,\infty}$ of the rate parameter for the rapid one of these transients was determined at different pH between 8 and 10, using $[4\text{-}^3\text{H}]\text{NADH}$ to slow down the reaction rates [3]. The results are given in Table 1, from which it can be seen that there is no pronounced variation of $r_{2,\infty}$ with pH under such conditions.

DISCUSSION

Interpretation of the Transient-State Rate Parameters for Alcohol Oxidation

Enzymes operating by a ternary-complex mechanism for the catalytic conversion of two substrates into two products contain two separate substrate-

Table 1. Effect of pH on the transient rate of benzaldehyde reduction in the liver alcohol dehydrogenase system

Estimates of $r_{2, \infty}$ were obtained by extrapolation to infinite substrate concentration of rate parameters r_2 for the rapid one of the two exponential transients observed at 328 nm on reacting $[4\text{-}^3\text{H}]\text{NADH}$ (100 μM) with varied concentrations of benzaldehyde (0.05–5 mM) at 25 °C in the presence of enzyme (about 8 μM) and pyrazole (20 mM)

pH	$r_{2, \infty}$
	s^{-1}
7.9	150 (± 35)
8.75	150 (± 25)
10.0	130 (± 20)

binding sites, each of which may be either unoccupied or occupied by the respective substrate or the corresponding product. A generalized ternary-complex mechanism, therefore, should account for the possible presence of four binary and four ternary enzymic complexes in addition to free enzyme. This leads to a kinetic scheme (a nine-center model) involving minimally 12 bimolecular and 14 monomolecular reaction steps. Depending on the relative magnitude of the corresponding rate constants, however, some reaction steps may be kinetically insignificant over the examined ranges of substrate and product concentrations. For this reason, the application of less complicated models for analysis of the kinetics of ternary-complex systems may be justified in certain cases and/or under certain experimental conditions.

In the case of liver alcohol dehydrogenase, the five-center model in Scheme 1 is known to provide an adequate description of the steady-state rate behaviour of the system over concentration ranges where no substrate inhibition is observed [1]. The transient-state kinetics of enzymic aldehyde reduction by NADH, also, can be well understood in view of this reaction scheme [4, 12]. For these reasons, our interpretation of the present and previously reported transient-state kinetic data for the enzymic oxidation of alcohols by NAD^+ will be based mainly upon the predictions of the compulsory-order mechanism in Scheme 1. It is recognized that this mechanism is too simple to account for the kinetic complications arising at inhibitory concentrations of the alcohol substrate [1]. The quantitative treatment of more complex reaction schemes (such six-center or seven-center models for ternary-complex reactions) will not be considered here, however, since such a treatment would provide no additional information about the process of ternary-complex interconversion.

The enzymic oxidation of NADH by aromatic aldehydes is known to be governed by two kinetically significant transients when monitored at 328 nm [4, 12]. As was shown in our previous analysis of

the kinetics inherent in Scheme 1, the appearance of two transients in such experiments requires that $k \gg k'$ and implies that only one exponential transient would be expected to be of kinetic significance for the reverse reaction of NAD^+ reduction [4, 12]. This is in obvious qualitative agreement with the observations made in the present and previous transient-state kinetic studies of the enzymic oxidation of alcohols [2, 3, 11, 16–20]. A single exponential transient has been observed for all alcohol substrates examined up to date, whether or not reactions have been carried out under single-turnover conditions. The present investigation, further, demonstrates that the rate behaviour of this transient is quantitatively consistent with Scheme 1 in all essential respects tested. The most convincing evidence in this direction is provided by the data in Fig. 3. These data show that the rate parameter r'_1 for benzyl alcohol oxidation under single-turnover conditions exhibits the dependence on substrate concentration predicted by Eqn (14), yielding a transient-state kinetic estimate of Φ'_2 which agrees excellently with that obtained by steady-state kinetic techniques. It can also be inferred from the latter observation that 100 mM isobutyramide prevents enzyme cycling without producing any additional kinetic effects that would obscure interpretation of the transient-state rate parameters observed.

According to Eqn (14), the dependence of r'_1 on substrate concentration c_P is hyperbolic and can be directly related to the magnitude of the steady-state kinetic coefficient Φ'_2 . The rate parameter r'_{1e} for the transient observed under cycling conditions exhibits a more complex dependence on c_P [Eqn (13)]. Although the data in Fig. 2 suggest that plots of $1/r'_{1e}$ vs $1/c_P$ might seem to be linear over limited ranges of substrate concentrations, it follows from Eqn (13) that the slope of the corresponding regression line cannot be given any obvious physical interpretation. Such slopes would, at the best, be expected to provide a rough estimate of the coefficient Φ'_2 . In any case, it should be emphasized that examination of the variation of r'_1 or r'_{1e} with substrate concentration cannot be assumed [3, 16, 19] to provide any direct information about the magnitude of the equilibrium constant for substrate-binding to the binary enzyme $\cdot \text{NAD}^+$ complex. The concentration dependence of the transient-state rate parameters should be related primarily to the coefficient Φ'_2 , the magnitude of which can be more conveniently and precisely determined by steady-state kinetic techniques.

This does not mean that examination of the concentration dependence of r'_1 or r'_{1e} is without practical interest. Besides being of value for testing the consistency between experiment and theory, such data can be used to obtain accurate estimates of the limiting values ($r'_{1, \infty}$ or $r'_{1e, \infty}$) of the rate parameters by extrapolation to infinite substrate concentration. The estimates of

$r'_{1,\infty}$ (16 s^{-1}) and $r'_{1c,\infty}$ (21 s^{-1}) thus obtained from the data in Fig. 2 and 3 establish that the transient rate of benzyl alcohol oxidation is higher under cycling conditions than under single-turnover conditions. As predicted by Eqn (16), further, the difference $r'_{1c,\infty} - r'_{1,\infty}$ agrees well in magnitude with the rate constant for coenzyme dissociation from the binary enzyme-NADH complex ($k_{-1} = 5.4 \text{ s}^{-1}$). This observation demonstrates the validity of the theoretical relationships derived in the present investigation, and confirms that the transient-state kinetics of enzymic alcohol oxidation can be quantitatively explained in view of Scheme 1.

The fact that transient rates of benzyl alcohol oxidation conform to Eqn (16) suggests that the condition $k'k_{-2} \gg (k_{-1})^2$ [under which Eqn (16) is valid] is fulfilled when benzyl alcohol is used as the substrate. Conclusive evidence in this direction is provided by the observation that $r'_{1,\infty}$ for benzyl alcohol exceeds k_{-1} by a factor of 3 which, according to Eqn (11), implies that $k'k_{-2}$ exceeds $(k_{-1})^2$ by a factor larger than $(2 \cdot 3)^2 = 36$. It can be similarly shown that Eqn (16) must be valid also for the enzymic oxidation of ethanol; according to the data reported by Brooks et al. [2], $r'_{1c,\infty}$ for ethanol greatly exceeds k_{-1} over the entire range of pH values tested. Application of Eqn (16) then leads to the conclusion that k_{-1} cannot contribute significantly to the magnitude of $r'_{1c,\infty}$ for ethanol. The data obtained under cycling conditions by Brooks et al. can be assumed to represent determinations of the rate parameter $r'_{1,\infty}$ and should be directly comparable to rate determinations carried out with saturating concentrations of aromatic substrates under single-turnover conditions.

The rate parameter $r'_{1,\infty}$, which is subject to a primary isotope effect when deuteriated alcohols are used as substrates, has been generally assumed to provide a direct measure of the rate constant k' for hydride transfer from the alcohol substrate to NAD^+ . This assumption appears to be valid as concerns the enzymic oxidation of ethanol; $r'_{1,\infty}$ for ethanol exhibits a constant isotope effect of about 6 over a range of pH values where $r'_{1,\infty}$ changes most significantly in magnitude [2]. The validity of such an assumption has to be tested for each individual substrate, however, by analysis of the full expression for $r'_{1,\infty}$ in Eqn (11). It cannot be excluded that the aldehyde dissociation step [k_{-2} in Eqn (11)] may contribute significantly to the limitation of $r'_{1,\infty}$ in some reactions.

In the case of benzyl alcohol/benzaldehyde catalysis at pH 8.75, it appears well established that $k \gg k'$ [12] and that both k and k' are subject to an isotope effect exceeding 2.5 [3,7,21]. On the other hand, there seems to be no pronounced isotope effect on the parameter (K_{app}) which defines the approximately hyperbolic dependence on substrate concentration of the transient-state rate parameter r_2 for benzalde-

Table 2. Rate constants in Scheme 1 for benzyl alcohol/benzaldehyde catalysis by liver alcohol dehydrogenase at pH 8.75

Estimates of k_1 and k'_1 have been taken from the report of DeTraglia et al. [9]. Values of other rate constants have been selected to provide the best fit to the kinetic parameter estimates listed in Table 3

Rate constant	Estimate	Rate constant	Estimate
	s^{-1}		$\text{s}^{-1} \mu\text{M}^{-1}$
k_{-1}	5.4	k_1	7
k'_{-1}	12	k'_1	0.9
k_{-2}	2500	k_2	5.6
k'_{-2}	11	k'_2	0.23
k	291		
k'	18		

hyde reduction [21]. The parameter K_{app} has been shown [4] to be given by

$$K_{app} = \frac{k + k_{-2}}{k_2} \quad (20)$$

The absence of an isotope effect on K_{app} , therefore, provides direct evidence that k_{-2} is considerably larger than k . This means that $k_{-2} \gg k \gg k'$, and under such conditions Eqn (11) reduces approximately to

$$r'_{1,\infty} = k' \quad (21)$$

It may be concluded that ternary-complex interconversion does represent the main rate-limiting step in the transient oxidation of benzyl alcohol at pH 8.75, as has been previously assumed. Whether or not Eqn (21) is valid also for other aromatic alcohols remains to be shown. The fact that significant isotope effects on K_{app} have been observed for several aromatic substrates tested [21] indicates that the validity of Eqn (21) should not be taken for granted.

The kinetics of benzyl alcohol oxidation and benzaldehyde reduction in the liver alcohol dehydrogenase system have been sufficiently well characterized to justify a detailed interpretation of the results in terms of rate constants in Scheme 1. Table 2 lists the set of rate constants which gives the best fit to the steady-state and transient-state kinetic parameter estimates reported by us for the reactions at pH 8.75 (Table 3). Estimates of rate constants for coenzyme binding have been taken from the data of DeTraglia et al. [9] and are used to show that the values proposed in Table 2 are consistent with the overall equilibrium constant (K) reported for the reaction [5]. Values indicated in Table 2 would, in general, be expected to be precise within 15%. Due to the low precision of the estimates of some kinetic parameters (K_{app} and $r_{2,\infty}$), however, there is a correspondingly larger uncertainty as concerns the precise magnitude of certain rate constants (k , k_{-2} , and k_2). Nevertheless, the data

Table 3. Kinetic parameters for benzyl alcohol/benzaldehyde catalysis by liver alcohol dehydrogenase at pH 8.75

Parameter estimates observed experimentally, compared with those calculated from the data in Table 2 using the theoretical relationships derived for Scheme 1. Symbols without primes refer to the enzymic reduction of benzaldehyde, and symbols with primes to the reverse reaction of benzyl alcohol oxidation

Parameter	Unit	Observed	Calculated	Reference
$r'_{1,\infty}$	s^{-1}	16	16	Eqn (11)
$r'_{10,\infty}$	s^{-1}	21	21	Eqn (15)
$r_{1,\infty}$	s^{-1}	10	10	[4]
$r_{2,\infty}$	s^{-1}	320	320	[4]
$A'_{1c,\infty}$		0.51	0.52	Eqn (18)
K_{app}	mM	0.4	0.5	[4]
Φ'_0	s	0.26	0.25	Eqn (10)
Φ_0	s	0.18	0.18	[4]
Φ'_1	$s \cdot \mu M$	7.4	7.3	Eqn (9)
Φ_2	$s \cdot \mu M$	4.2	4.2	[4]
K	M	$\approx 4 \times 10^{-11}$	6×10^{-11}	[5]

in Tables 2 and 3 establish that magnitudes of all transient-state rate parameters for the system can be consistently accounted for in terms of the relationships derived [4] for the compulsory-order mechanism in Scheme 1.

Amplitudes of the Exponential Transient

In view of previous suggestions that liver alcohol dehydrogenase exhibits a half-of-the-sites reactivity [1], it might be adequate to comment briefly upon the amplitude of the exponential transient observed during the enzymic oxidation of alcohols by NAD^+ . As indicated by the approximate Eqn (19), the amplitude of the burst of NADH production would be expected to approach the active site concentration of enzyme when $k' \gg k_{-1}$. This explains why an approximately full burst is always observed under conditions such that the apparent rate of coenzyme dissociation is low in comparison to the rate of ternary-complex interconversion (e.g. in reactions performed under single-turnover conditions or at strongly inhibitory substrate concentrations, and in reactions involving ethanol or other substrates for which $k' \gg k_{-1}$). Similarly, Eqn (19) indicates that burst amplitudes lower than c_E will be obtained in reactions carried out under cycling conditions with non-inhibitory concentrations of those alcohols for which the magnitude of k' is comparable to, or lower than, that of k_{-1} . This explains the observed attenuation of the burst amplitude for aromatic alcohols, as well as the absence of a detectable burst in reactions (e.g. those involving methanol and 2-propanol [18]) where hydride transfer from the alcohol to NAD^+ represents the main rate-limiting step.

Application of the more precise Eqn (18) to the data in Table 2 shows that $A'_{1c,\infty}$ for benzyl alcohol oxidation would be expected to equal $0.52 c_E$ at pH 8.75. This agrees excellently with the saturation value of $0.51 c_E$ observed experimentally by us and by Baici et al. [11] for non-inhibitory substrate concentrations. Since $r'_{1,\infty}$ (Fig. 3) and k_{-1} [9] exhibit no pronounced variation with pH between 7 and 9, further, it follows from Eqns (11) and (18) that the burst amplitude for benzyl alcohol oxidation should remain essentially constant within this pH range. The observation that benzyl alcohol oxidation under cycling conditions at pH 7–9 produces a transient burst of NADH formation corresponding to approximately one half of the active site concentration of enzyme [11], therefore, cannot be taken as evidence in favour of a half-of-the-sites reactivity of the enzyme. The transient-state kinetics of alcohol oxidation are, in all respects tested, qualitatively and quantitatively consistent with an independent action of identical enzymic sites according to the compulsory-order mechanism in Scheme 1, except for the obvious fact that the effects caused by inhibitory concentrations of substrate have to be explained in view of a more complex mechanism (a six-center or seven-center model) accounting for the substrate-inhibition phenomenon.

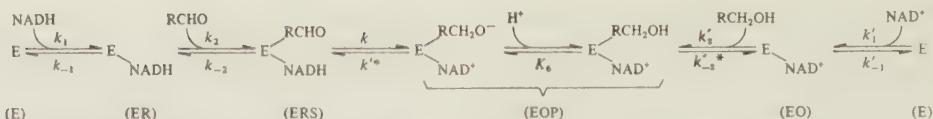
pH Dependence of the Process of Ternary-Complex Interconversion

The data in Tables 2 and 3 justify application of the approximation

$$r_{2,\infty} = k \quad (22)$$

for interpretation of the transient-state rate parameter $r_{2,\infty}$ for benzaldehyde reduction at pH values around 9, or more precisely, Eqn (22) is valid as long as $k \gg k' + k'_{-2}$ [4]. The data in Table 1, further, establish that $r_{2,\infty}$ for benzaldehyde reduction remains essentially constant over the range of pH values (8–10) where reliable determinations of this rate parameter can be performed. The present results, therefore, lend support to the conclusion of McFarland et al. [3] that there is no pronounced effect of pH on the rate of hydride transfer from NADH to aromatic substrates.

On the other hand, we have to reject the conclusion of McFarland et al. [3] that there is no specific effect of pH on the rate parameter $r'_{1,\infty}$ for benzyl alcohol oxidation. The data in Fig. 4 (which actually agree well with those reported by McFarland et al. over the more limited pH range examined by these authors) establish that $r'_{1,\infty}$ is dependent on a proton dissociation step with a pK_a of 6.4. Since both k_{-2} [22, 23] and k [3] in Eqn (11) would be expected to remain unaffected by pH changes, the approximate relationship for $r'_{1,\infty}$ in Eqn (21) appears to be valid throughout



Scheme 2. Reaction mechanism proposed to account for the asymmetric pH dependence of the process of ternary-complex interconversion in the liver alcohol dehydrogenase system

the pH range studied (pH 5–9). The variation of $r'_{1,\infty}$ with pH, therefore, cannot be attributed to a change in rate-limiting step, but must be assumed to reflect a pH dependence of the rate constant k' for ternary-complex interconversion (Scheme 1). This can be inferred also from the observation that there is no decrease in the isotope effect on $r'_{1,\infty}$ when the pH is lowered from 8.75 to 5.9 [3], while the magnitude of the parameter decreases by a factor of 4 (Fig. 4). The fact that a similar pH dependence has been reported for the transient rate of ethanol oxidation [2] lends strong support to the above interpretation of the data in Fig. 4, and indicates that the pK_a 6.4 dependencies observed have a common mechanistic origin.

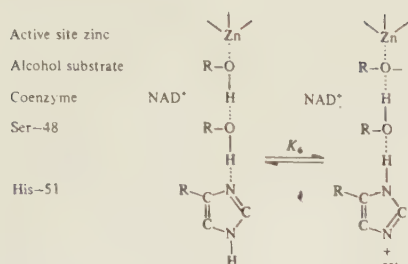
The present investigation, therefore, provides direct evidence that rate constants k and k' for ternary-complex interconversion (as defined in Scheme 1) exhibit an asymmetric dependence on pH; the absence of an effect of pH on k over the pH range 8–10 excludes that aldehyde reduction is subject to any apparent acid catalysis corresponding to the apparent base catalysis of alcohol oxidation indicated by the pK_a 6.4 dependence of k' . According to the principle of microscopic reversibility, this means that the pK_a 6.4 dependence of k' cannot be attributed to an effect on the actual step of hydride transfer. The pH dependence of k' must be related to a proton dissociation reaction involving only one (EOP) of the ternary complexes in Scheme 1. The observation that k' tends towards zero at low pH (Fig. 4) provides evidence that this proton dissociation reaction may be considered to represent an obligatory step in the catalytic mechanism.

These implications of the present results suggest that Scheme 1 for the catalytic action of liver alcohol dehydrogenase must be extended to include minimally the protonation equilibrium inserted in Scheme 2. This extension leads to a formal correction of the reaction scheme; Scheme 2 (in contrast to Scheme 1) adequately accounts for the stoichiometry and the electro-neutrality of the catalytic reaction. Moreover, the extended reaction scheme makes perfect sense from a mechanistic point of view. Scheme 2 has been written in a form which draws attention to the fact that hydride transfer from NADH to an aldehyde substrate results in formation of the corresponding alcoholate ion and NAD^+ . According to what is known about the tertiary structure of the enzyme [1],

the negatively charged alcoholate ion must be assumed to be bound in a highly hydrophobic environment and in proximity to both NAD^+ and the Zn^{2+} ion at the catalytic center of the protein. Coulombic interactions, therefore, will render the rate of dissociation of the alcoholate ion negligibly small in comparison to the rate of dissociation of the uncharged alcohol. This means that proton transfer to the alcoholate ion (required to account for the catalytic alcohol production and proton consumption) must be expected to represent an obligatory reaction step preceding product release, as indicated in Scheme 2. Since such a proton transfer can be assumed to be rapid in comparison to other reaction steps in the mechanism, a distinction between Scheme 1 and Scheme 2 becomes of kinetic interest only for interpretation of the effect of pH on the catalytic process. In the latter context, k' in Scheme 1 should be treated as an apparent rate constant for hydride transfer, being related to the 'true' rate constant k'^* in Scheme 2 through

$$k' = \frac{k'^*}{1 + \frac{[H^+]}{K_6}} \quad (23)$$

When interpreted in view of Scheme 2, the present results lead to the conclusion that there is no detectable effect of pH on the actual process of hydride transfer (no effect on the rate constants k and k'^*). The pH dependence of the apparent rate constant k' for hydride transfer from alcohol substrates to NAD^+ is consistent with Eqn (23) and appears to derive from a proton-dependent pre-equilibration step occurring at the ternary-complex level. Application of Eqn (23) to the experimental data reported for the oxidation of ethanol [2] and benzyl alcohol (Fig. 4) gives agreeing estimates ($pK_6 = 6.4$) of the acid dissociation constant for this pre-equilibration step. It cannot be excluded, however, that pK_6 may vary in magnitude depending on the electronic properties of the alcohol substrate. The latter possibility becomes evident on consideration of Scheme 2, according to which the pre-equilibration step can be envisaged to represent proton dissociation from the enzyme-bound alcohol to form the corresponding alcoholate ion. This would mean that the apparent pK_a of the alcohol substrate is drastically decreased (from about 17 to below 7) on formation of the ternary enzyme $\cdot NAD^+ \cdot \text{alcohol}$ complex. Factors which might contribute



Scheme 3. Proton dissociation equilibrium suggested to be responsible for the pK_a -6.4 dependence of the transient rate of alcohol oxidation in the liver alcohol dehydrogenase system

to such a pK_a perturbation will be discussed in a later report dealing with the pH dependence of substrate-binding to the enzyme. It may suffice to point out here that perturbation of the pK_a of the alcohol to a value below 7 in the productive ternary complex would be mechanistically sound; it could be envisaged to have the obvious catalytic function of facilitating hydride transfer to NAD^+ by ensuring that the substrate is bound as an alcoholate ion at physiological pH.

Strong evidence has accumulated during the last five years indicating that the oxygen of aldehyde and alcohol substrates coordinates to the catalytic zinc atom in liver alcohol dehydrogenase on substrate-binding to the enzyme [21–23]. As has been pointed out by Brändén et al. [1], proton transfer from the solution to a zinc-bound oxygen would be expected to be mediated by the side chains of His-51 and Ser-48 through a system of hydrogen bonds. It may then be noted that the present results imply that catalytic ternary-complex interconversion is associated with proton transfer to or from the reaction solution; internal proton exchange between the enzyme-bound substrate and ionizing groups in the protein (or zinc-bound water) would not be dependent on the pH of the solution, i.e. could not lead to any effect of pH on k' . This means that the His-51/Ser-48 proton transfer system must be expected to participate in the proton dissociation step inserted in Scheme 2. In other words, it seems likely that the observed pK_a 6.4 dependence of k' for ethanol and benzyl alcohol oxidation should be attributed to a coupled acid-base system, such as the one indicated in Scheme 3, rather than to an isolated alcohol/alcoholate system. The possibility that the coupled system involves also zinc-bound water is not ruled out by the present results.

The present investigation is intended to be part of a systematic study of the pH dependence of liver alcohol dehydrogenase catalysis. In future reports we will show how the reaction mechanism proposed in the minimal Schemes 2 and 3 can be reconciled with the observed pH dependence of the binding of substrate and coenzyme to the enzyme, as well as with the kinetics of proton release or uptake during catalysis.

This investigation was supported by grants from the Swedish Natural Science Research Council.

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Effect of pH on the Binding of Decanoate and Trifluoroethanol to Liver Alcohol Dehydrogenase

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1. Rate constants for dissociation of the substrate analogues decanoate and trifluoroethanol from the respective ternary complex formed with liver alcohol dehydrogenase and NAD^+ have been determined at different pH. The rate of decanoate dissociation exhibits no pronounced dependence on pH. The trifluoroethanol dissociation rate is regulated by a proton equilibrium with a $\text{p}K_a$ of 4.3, alcohol desorption occurring exclusively from the protonated form of the ternary complex.

2. In contrast to what has been previously suggested from equilibrium binding studies, these observations provide evidence that the two substrate analogues combine to the same, protonated, form of the binary enzyme $\cdot \text{NAD}^+$ complex. This means that catalytic proton release during the enzymic oxidation of alcohol substrates is likely to take place at the ternary-complex level. It is shown that proton release coupled to the catalytic hydride transfer step can be readily detected at the appropriate pH during the enzymic oxidation of benzyl alcohol.

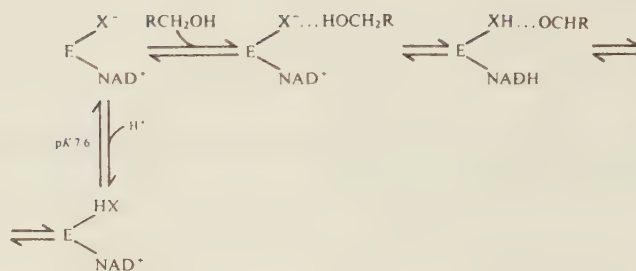
3. The $\text{p}K_a$ -4.3 dependence of trifluoroethanol dissociation is suggested to reflect an alcohol/alcoholate ion equilibration of the enzyme-bound substrate analogue. Substitution of trifluoroethanol by benzyl alcohol in the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex results in an uptake of close to one proton per active site of the enzyme at pH 5.7. This observation is consistent with our previous proposal that the enzyme $\cdot \text{NAD}^+$ $\cdot$ benzyl alcohol complex participates in a similar alcohol/alcoholate ion equilibrium with a $\text{p}K_a$ of 6.4.

It has been well established that NAD^+ binding to liver alcohol dehydrogenase perturbs the $\text{p}K_a$ of an ionizing group on the enzyme from a value above 9 in free enzyme to 7.6 in the binary enzyme $\cdot \text{NAD}^+$ complex [1]. Shore et al. [2] have drawn attention to the role of this ionizing group in the process of substrate binding to the enzyme. Titrimetric and kinetic evidence was presented showing that trifluoroethanol (decanoate) binding to the enzyme $\cdot \text{NAD}^+$ complex is dependent on the ionization state of the $\text{p}K_a$ -7.6 group, with apparent tight binding to the unprotonated (protonated) form. These observations were taken to indicate that trifluoroethanol and, by analogy, alcohol substrates bind directly to the basic form of the $\text{p}K_a$ -7.6 group, while decanoate and aldehyde substrates combine to the acid form of the group. A reaction mechanism consistent with such a pattern of substrate binding was proposed (Scheme 1), according to which the $\text{p}K_a$ -7.6 group has a crucial function as a mediator

of proton transfer from substrate to solution during catalysis. The latter interpretation of the experimental data presented by Shore et al. appears to have been generally accepted, and the idea is now widely held that proton release to solution during the enzymic oxidation of alcohols takes place at the binary-complex level as an obligatory reaction step following upon NAD^+ binding [1].

It should be noted, however, that effects of pH on the reactivity of the enzyme $\cdot \text{NAD}^+$ complex in a binding process are reflected only by the on-velocity constant, while the corresponding equilibrium binding constant may reflect also effects of pH on the off-velocity constant, i.e. on the reactivity of the ternary complex formed. The proposal of Shore et al. [2], therefore, is based on the (unexpressed) assumption that the observed pH dependence of the equilibrium constant for trifluoroethanol and decanoate binding derives exclusively from the on-velocity constant. Unfortunately, no evidence is available which could justify such an assumption, for which reason the validity of Scheme 1 remains uncertain. To eliminate

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).



Scheme 1. Reaction mechanism proposed by Shore et al. [2] for alcohol binding and proton transfer during liver alcohol dehydrogenase catalysis

this uncertainty, we have carried out a complementary characterization of the effect of pH on trifluoroethanol and decanoate binding to the enzyme $\cdot$ NAD^+ complex. The results obtained call for a reinterpretation of the important binding data reported by Shore et al., and an alternative mechanism for substrate binding and proton transfer during liver alcohol dehydrogenase catalysis is presented.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse-liver alcohol dehydrogenase from Boehringer Mannheim GmbH was further purified as described by Bernhard et al. [3]. When required, unbuffered enzyme solutions were prepared using 33 mM sodium sulphate for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [4] and are reported throughout as active-site concentrations.

The coenzymes NAD^+ and NADH (Sigma Chemical Corp.; grade III) were purified as described by Gurr et al. [5]. Pyrazole (Fluka AG) was recrystallized twice from water prior to use. Other reagents were of analytical grade and were used without further purification.

All experiments reported in the present paper were performed at 25°C in buffer or sodium sulphate solutions of 0.1 M ionic strength, prepared using water twice distilled from a quartz apparatus.

Rapid-Reaction Equipment and Data Processing

Kinetic measurements were made photometrically by stop-flow techniques, using the rapid-reaction equipment described previously [6]. Rates and amplitudes of the exponential transient observed were estimated by non-linear regression analysis as detailed previously [6].

Dissociation Rate Constant Determinations

Rate constants for dissociation of the substrate analogues decanoate and trifluoroethanol from the respective ternary complex formed with liver alcohol dehydrogenase and NAD^+ were determined by displacement methods [7]. The ternary complex was prepared by premixing the enzyme ($10\text{--}20\text{ }\mu\text{M}$) in one syringe with NAD^+ (usually 4 mM) and an at least 90% saturating concentration of the substrate analogue according to the binding data reported by Shore et al. [2]. The second syringe contained varied concentrations of pyrazole (about $0.1\text{--}2\text{ mM}$ at high pH and $10\text{--}200\text{ mM}$ at low pH), sufficiently high to cause an extensive displacement of the substrate analogue from the ternary complex after mixing in the stop-flow apparatus. Apparent first-order rate constants for the displacement process were determined at 297 nm where the enzyme $\cdot$ NAD^+ $\cdot$ pyrazole complex provides a major contribution to the absorbance changes observed. Control experiments performed in the absence of substrate analogues established that no first-order process slower than 1000 s^{-1} limits the rate of pyrazole association to the enzyme $\cdot$ NAD^+ complex over the investigated pH range.

Reactions at pH 6–9 were performed in phosphate buffer solutions by conventional techniques, i.e. by mixing buffered reactant solutions of equal pH. To avoid extensive denaturation of the enzyme through premixing at low pH, a different procedure was used to initiate reactions at pH values below 6. Preincubation of the enzyme with NAD^+ and the substrate analogue was carried out at pH 6 in a 33 mM solution of sodium sulphate. The displacement process was then initiated by mixing the unbuffered solution containing the enzymic ternary complex with pyrazole dissolved in succinate buffer (pH 4–6) or citrate buffer (below pH 4). The pH of the reaction solution after mixing was determined in control experiments and was found not to differ significantly from that of the buffered pyrazole solution used.

No detectable denaturation of the enzyme took place during the short period of time (less than 1 s

at low pH) considered in the displacement experiments. Examination of the 297-nm absorbance changes occurring over a prolonged period of time indicated that the enzyme · NAD⁺ · pyrazole complex formed through the displacement reaction decomposed at a significant rate below pH 5. The rate of decomposition of the complex was at least two orders of magnitude lower than the rate of its formation, and reliable estimates of apparent first-order rate constants for the displacement process could be obtained down to pH 3.4.

Proton Release Measurements

Stop-flow kinetic methods analogous to those described by Dunn [8] and Shore et al. [2] were used to measure proton release/uptake in experiments where the enzyme · NAD⁺ complex or the enzyme · NAD⁺ · trifluoroethanol complex was reacted with a large excess of benzyl alcohol. Premixing and reactions were performed at pH 5.7 in 33 mM sodium sulphate solutions buffered with 60 μ M chlorophenol red. Steady-state kinetic control experiments indicated that the presence of the pH indicator had no significant effect on the catalytic activity of the enzyme.

Absorbance changes during reaction were monitored at 574 nm (proton consumption/production) or at 320 nm (NADH production). The amount of NADH produced was calculated using a difference absorption coefficient of 4900 cm⁻¹ M⁻¹ at 320 nm (the isosbestic point for free and enzyme-bound NADH under the actual experimental conditions). A calibration value for the 574-nm absorbance changes brought about by proton release/uptake was determined with a Zeiss PM QII spectrophotometer by adding known amounts (5–20 μ M) of HCl to a cuvette containing all the components of the final reaction solution except for benzyl alcohol. The calibration value thus obtained (see legend to Fig. 4) was found to agree with that calculated from the steady-state decrease in 574-nm absorption during reaction [2], as well as with that determined from the increase in 574-nm absorption observed on displacement of NAD⁺ from the enzyme · NAD⁺ · trifluoroethanol complex with a large excess of NADH (4 mM). The latter reaction was assumed to result in an uptake of one proton per mol NAD⁺ displaced at pH 5.7 [2].

RESULTS

Trifluoroethanol Binding

Eqn (1) shows the reaction scheme for trifluoroethanol (RCH₂OH) binding to the binary complex formed between liver alcohol dehydrogenase (E) and NAD⁺.

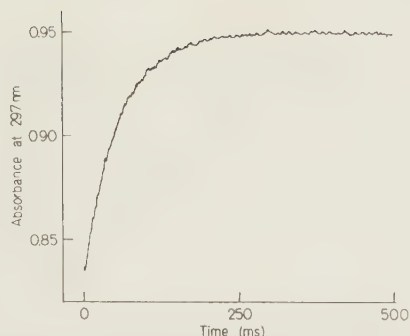
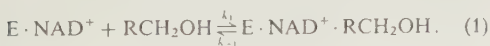


Fig. 1. Displacement of trifluoroethanol with pyrazole from the ternary complex formed with liver alcohol dehydrogenase and NAD⁺. Absorbance changes recorded at 297 nm and 25 °C on mixing enzyme (20 μ M), preincubated at pH 6.0 with NAD⁺ (4 mM) and trifluoroethanol (6 mM) in 33 mM sodium sulphate, with a 0.1 M ionic strength succinate buffer solution of pyrazole (100 mM). The pH of the reaction solution after mixing was 5.7. The transient observed exhibits an apparent first-order rate constant of 18 s⁻¹.

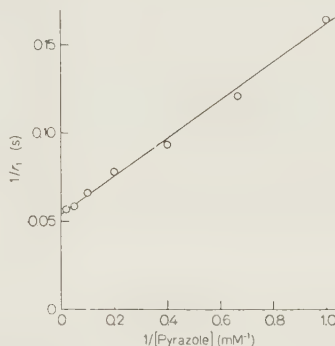


Fig. 2. Dependence on pyrazole concentration of the apparent first-order rate constant (r_1) for displacement of trifluoroethanol with pyrazole. Conditions as in Fig. 1, except that the concentration of pyrazole was varied. The intercept of the regression line drawn corresponds to a trifluoroethanol dissociation rate constant of 19 s⁻¹.

The off-velocity constant (k_{-1}) in Eqn (1) was determined at different pH between 3.4 and 9 by displacement of trifluoroethanol with varied concentrations of pyrazole. Formation of the enzyme · NAD⁺ · pyrazole complex was monitored by stop-flow techniques at 297 nm.

Fig. 1 shows the absorbance changes recorded at 297 nm during a typical displacement experiment. The apparent first-order rate constant (r_1) for the single-exponential transient observed was found to exhibit a hyperbolic dependence on the concentration of pyrazole. This is illustrated in Fig. 2 by examples of the measurements made at pH 5.7. In accordance with the kinetic theory for displacement reactions [7], the

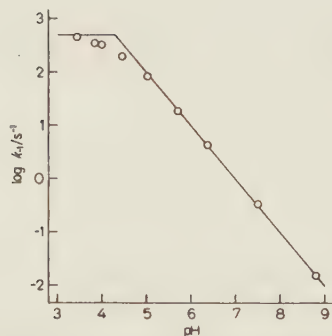


Fig. 3. Effect of pH on the rate constant for trifluoroethanol dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Experimental points fall along a monobasic dissociation curve with a pK_4 of 4.3

intercept of the straight line obtained on plotting $1/r_1$ vs $1/[\text{pyrazole}]$ (Fig. 2) was taken to provide a measure of the reciprocal value of k_{-1} . The estimates of k_{-1} thus obtained at different pH are given in Fig. 3.

The results in Fig. 3 show that the magnitude of k_{-1} is approximately proportional to the hydrogen ion concentration above pH 5.5. At lower pH k_{-1} tends towards a limiting value of 500 s^{-1} , suggesting that the off-velocity constant for trifluoroethanol binding conforms to a monobasic dissociation function of the form.

$$k_{-1} = \frac{k_{-1}^*}{1 + \frac{K_4}{[\text{H}^+]}} \quad (2)$$

The parameter estimates obtained on fitting Eqn (2) to the data in Fig. 3 were $k_{-1}^* = 500 (\pm 100) \text{ s}^{-1}$ and $\text{pK}_4 = 4.3 (\pm 0.2)$.

Shore et al. [2] found that the pH dependence of the equilibrium dissociation constant ($K_1 = k_{-1}/k_1$) for complex formation according to Eqn (1) can be expressed as

$$K_1 = K_1^* \left(1 + \frac{[\text{H}^+]}{K_7} \right) \quad (3)$$

between pH 5.5 and 8.5, with $\text{pK}_7 = 7.6$ and $K_1^* = 4 \mu\text{M}$. Since Eqn (2) reduces approximately to

$$k_{-1} = \frac{k_{-1}^* [\text{H}^+]}{K_4} \quad (4)$$

above pH 5.5, it follows from Eqns (2) and (3) that the pH dependence of the on-velocity constant for trifluoroethanol binding is given by

$$k_1 = \frac{k_1^*}{1 + \frac{K_7}{[\text{H}^+]}} \quad (5)$$

Table 1. Effect of pH on the rate constant for decanoate dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+

pH	Rate constant (k_{-2})	
	s^{-1}	
5.5	4.5	(± 1.0)
6.4	2.4	(± 0.4)
7.0	2.1	(± 0.2)
8.0	1.9	(± 0.3)

over the pH range investigated by Shore et al. The value of k_1^* corresponding to the above estimates of k_{-1}^* , K_1^* , pK_4 , and pK_7 is $k_1^* = 0.06 \text{ s}^{-1} \mu\text{M}^{-1}$.

Decanoate Binding

The off-velocity constant for decanoate (RCOO^-) binding according to Eqn (6) was determined by methods entirely analogous to those described for trifluoroethanol binding.

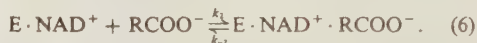


Table 1 shows the estimates of k_{-2} obtained at different pH. The results provide clear evidence that there is no pronounced effect of pH on the magnitude of k_{-2} over the investigated pH range (5.5–8).

Shore et al. [2] found that the pH dependence of the equilibrium constant (K_2) for decanoate dissociation from the ternary complex in Eqn (6) can be expressed as

$$K_2 = K_2^* \left(1 + \frac{K_7}{[\text{H}^+]} \right) \quad (7)$$

with $\text{pK}_7 = 7.6$ and $K_2^* = 2 \mu\text{M}$. Assuming that $k_{-2} = 2 \text{ s}^{-1}$ (Table 1) it may be concluded from Eqn (7) that the pH dependence of the on-velocity constant for decanoate binding is given by

$$k_2 = \frac{k_2^*}{1 + \frac{K_7}{[\text{H}^+]}} \quad (8)$$

between pH 5.5 and 8 with $\text{pK}_7 = 7.6$ and $k_2^* = 1 \text{ s}^{-1} \mu\text{M}^{-1}$.

Proton Transfer at the Ternary-Complex Level

NADH production during the catalytic action of liver alcohol dehydrogenase can be conveniently monitored at the isobestic point for free and enzyme-bound NADH (320 nm at pH 5.7). The lower trace in Fig. 4 shows the 320-nm absorbance changes re-

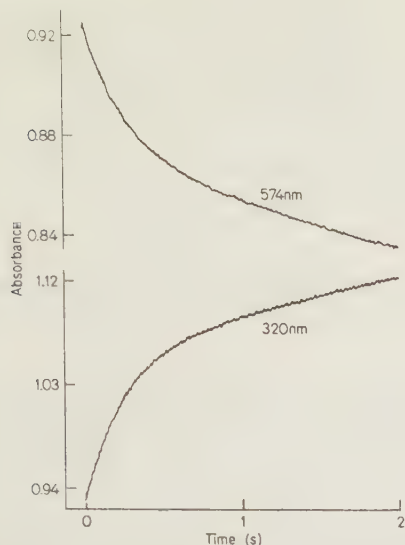


Fig. 4. NADH production and proton release in the reaction between benzyl alcohol and the binary enzyme · NAD⁺ complex. Absorbance changes recorded at 320 nm (lower trace) and 574 nm (upper trace) on mixing liver alcohol dehydrogenase (30 μ M), preincubated with NAD⁺ (6 mM), with benzyl alcohol (150 mM) at 25°C in 33 mM sodium sulphate buffered with 60 μ M chlorophenol red (pH 5.7). A release of one proton per active site of the enzyme corresponds to an increase of 0.076 in 574-nm absorbance

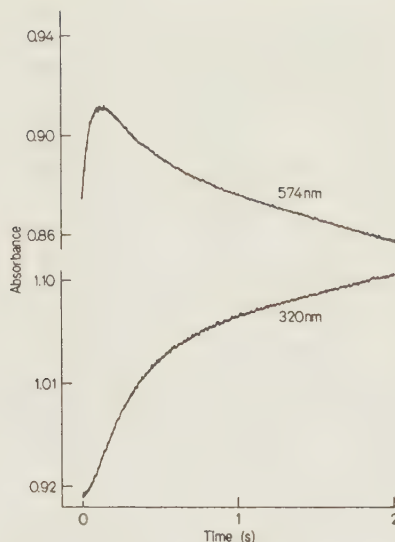


Fig. 5. NADH production and proton uptake/release in the reaction between benzyl alcohol and the ternary enzyme · NAD⁺ · trifluoroethanol complex. Conditions as in Fig. 4, except that the enzyme was preincubated with 6 mM NAD⁺ and 8 mM trifluoroethanol

corded on reacting the enzyme · NAD⁺ complex with 75 mM benzyl alcohol at pH 5.7. In accordance with previous reports [6,9–11], the reaction exhibits a transient burst of NADH production prior to the attainment of steady-state conditions. It has been well established that this transient reflects formation of the enzyme · NADH · benzaldehyde complex in the catalytic hydride transfer step, and the rate (3.7 s^{-1}) and amplitude (0.85 mol NADH per active site of the enzyme) of the burst in Fig. 4 agree well with those expected according to previously reported data for the reaction [6]. The upper trace in Fig. 4 shows the 574-nm absorbance changes due to proton release during the same reaction. The results demonstrate that the transient production of NADH through ternary-complex interconversion in the hydride transfer step is coupled to a release of 0.7 active-site-equivalent of protons at pH 5.7, i.e. to a release of approximately 0.8 proton/NADH molecule formed. Proton liberation in the subsequent steady-state phase exhibits the expected stoichiometry of 1 mol/mol NADH produced.

The lower trace in Fig. 5 shows the 320-nm absorbance changes recorded on reacting the enzyme · NAD⁺ · trifluoroethanol complex with 75 mM benzyl alcohol at pH 5.7. The upper trace shows the kinetics

of proton uptake/release during the same reaction. Concentrations of reactants in this displacement experiment were such that the productive enzyme · NAD⁺ · benzyl alcohol complex would be expected to be formed at a rate limited mainly by the rate of trifluoroethanol dissociation from the initial ternary complex [7]. At pH 5.7, the latter rate is high (19 s^{-1} , Fig. 3) in comparison to the rate of hydride transfer in the productive ternary complex formed (3.7 s^{-1} , Fig. 4). This explains why 320-nm absorbance changes during the reaction in Fig. 5 are governed mainly by a single transient exhibiting approximately the same amplitude and rate as the one observed in the reaction between the enzyme · NAD⁺ complex and benzyl alcohol (Fig. 4).

Examination of the upper trace in Fig. 5 shows that pH changes brought about by the displacement of trifluoroethanol with benzyl alcohol occur in three distinct phases. An initial rapid transient uptake of protons is followed by a slower transient release of protons prior to the attainment of a steady-state rate of proton liberation. The rapid one of the two transients exhibits a rate constant (18 s^{-1}) agreeing well with that of trifluoroethanol dissociation at pH 5.7. This transient, therefore, can be attributed to the actual displacement reaction leading to formation of

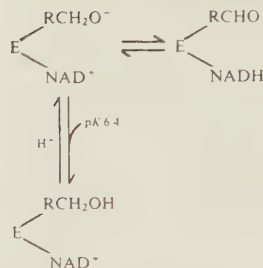
the productive enzyme $\cdot$ NAD $^{+}$ $\cdot$ benzyl alcohol complex. The amplitude of the rapid transient corresponds to an uptake of 0.8 proton/active site of the enzyme. The slower one of the two transients exhibits a rate constant (3.6 s^{-1}) and amplitude (0.7 active-site-equivalent of proton) agreeing well with those expected for proton release coupled to the transient NADH production in the hydride transfer step (Fig. 4; Fig. 5, lower trace). Proton liberation in the final steady-state phase exhibits a stoichiometry of one proton per NADH molecule formed.

Equilibrium calculations indicated that 96% (9%) of the enzyme can be assumed to be present as the enzyme $\cdot$ NAD $^{+}$ $\cdot$ trifluoroethanol complex at the start (end) of the transient part of the displacement reaction. The results in Fig. 5, therefore, provide evidence that substitution of trifluoroethanol by benzyl alcohol in the ternary complex results in an uptake of approximately 0.9 equivalent of proton at pH 5.7.

DISCUSSION

The equilibrium binding data presented by Shore et al. [2] establish that ternary-complex formation between liver alcohol dehydrogenase, NAD $^{+}$, and decanoate or trifluoroethanol is dependent on the ionization state of (the pK_a -7.6 group in) the binary enzyme $\cdot$ NAD $^{+}$ complex. The present results show that there is no significant effect of pH on the rate constant for decanoate dissociation from the ternary complex (Table 1). This means that the observed pH dependence of the equilibrium constant for decanoate binding to the enzyme $\cdot$ NAD $^{+}$ complex can be assumed to derive entirely from the on-velocity constant, i.e. to reflect only proton dissociation equilibria affecting the reactivity of the binary complex (it is assumed that proton transfer steps equilibrate rapidly in comparison to other reaction steps in the mechanisms and kinetic schemes considered in this discussion). The present investigation, therefore, provides evidence justifying the conclusion of Shore et al. [2] that decanoate binds preferentially to the protonated form of the enzyme $\cdot$ NAD $^{+}$ complex.

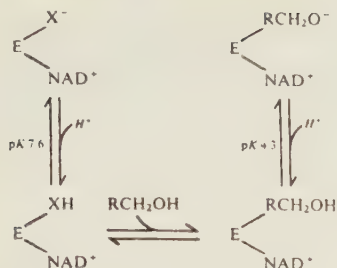
The data in Fig. 3, however, establish that the off-velocity constant for trifluoroethanol binding is strongly dependent on pH as expressed by Eqn (2). This means that the observed pH dependence of the equilibrium constant for trifluoroethanol binding reflects not only the protonation state of the enzyme $\cdot$ NAD $^{+}$ complex, but also that of the ternary complex formed. Taking the latter pH effect into consideration, it follows from the equilibrium data presented by Shore et al. [2] that the pH dependence of trifluoroethanol association [Eqn (5)] is entirely analogous to that of decanoate association [Eqn (8)]. The present results, therefore, invalidate the proposal of Shore



Scheme 2. Proton equilibrium suggested [6] to regulate the apparent rate of hydride transfer from benzyl alcohol to NAD $^{+}$.

et al. that trifluoroethanol binds to the unprotonated form of the enzyme $\cdot$ NAD $^{+}$ complex. The data now available for decanoate and trifluoroethanol binding provide clear evidence that both substrate analogues combine preferentially to the protonated form of the binary complex.

The observation that decanoate and trifluoroethanol dissociation rates exhibit distinct pH dependences calls for an explanation. We have inferred previously from the observed effect of pH on the enzymic process of ternary-complex interconversion [6] that the enzyme $\cdot$ NAD $^{+}$ $\cdot$ benzyl alcohol complex participates in a proton dissociation step exhibiting a pK_a of 6.4. It was pointed out that this reaction step can be formally regarded as an alcohol/alcoholate ion interconversion of the enzyme-bound substrate, and evidence was presented indicating that catalytic hydride transfer to NAD $^{+}$ occurs exclusively when the substrate is in the alcoholate ion form (Scheme 2). The results in Fig. 4 lend strong support to this idea. They show that hydride transfer during the enzymic oxidation of saturating concentrations of benzyl alcohol at pH 5.7 is directly coupled to a transient release of 0.8 proton/NADH molecule formed. This observation is qualitatively and quantitatively consistent with Scheme 2, according to which 0.83 proton would be expected to be liberated for every molecule of NADH produced during the transient phase of coenzyme reduction when the reaction is initiated at the ternary-complex level (assuming a pK_a of 6.4 for the enzyme $\cdot$ NAD $^{+}$ $\cdot$ benzyl alcohol complex, 83% of the complex is present in the protonated form at pH 5.7). In the actual experimental situation, the reaction is initiated by the addition of a large excess benzyl alcohol to the enzyme $\cdot$ NAD $^{+}$ complex. Proton release due to alcohol binding and subsequent alcohol/alcoholate ion equilibration of the enzyme-bound substrate, however, can be assumed to be completed within the dead-time of the stop-flow apparatus. This means that the experimentally observed proton release must derive from a proton dis-

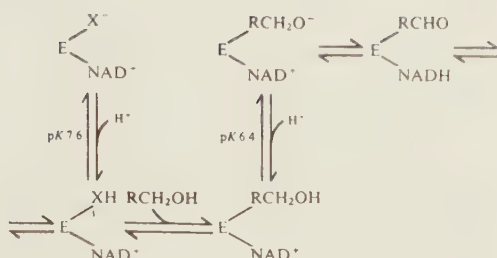


Scheme 3. Proton equilibria suggested to regulate the binding of trifluoroethanol to the enzyme · NAD⁺ complex

sociation equilibrium which involves the ternary complex and affects the reactivity of this complex as indicated in Scheme 2.

The pH dependence data in Fig. 3 and Eqn (2) provide evidence that the enzyme · NAD⁺ · trifluoroethanol complex participates in a similar acid-base equilibrium with a pK_a of 4.3, alcohol dissociation taking place exclusively from the protonated form of the ternary complex. Crystallographic data indicate that trifluoroethanol and alcohol substrates form structurally analogous ternary complexes [12]. It therefore seems reasonable to assume that the ternary complexes formed with trifluoroethanol and benzylalcohol participate in mechanistically analogous ionization processes, which suggests that the pK_a -4.3 dependence of trifluoroethanol dissociation can be formally attributed to an alcohol/alcoholate ion equilibration of the enzyme-bound substrate analogue (Scheme 3). This would explain why dissociation of the substrate analogue occurs only from the protonated form of the ternary complex; the negatively charged alcoholate ion would be expected to be considerably more tightly bound than the neutral alcohol due to electrostatic interaction with NAD⁺ and the catalytic zinc ion at the active center of the enzyme [1]. The shift in pK_a from 6.4 for enzyme-bound benzyl alcohol to 4.3 for enzyme-bound trifluoroethanol can be well understood in view of the difference in pK_a for ionization of the hydroxyl group in the free alcohol [13]. The fact that decanoate lacks a corresponding ionizing group implies that the enzyme · NAD⁺ · decanoate complex cannot participate in a similar acid-base equilibrium. This offers a reasonable explanation for the lack of effect of pH on the rate of decanoate dissociation from the ternary complex over the investigated pH range.

According to this interpretation of the experimental results, 96% (17%) of the enzyme-bound trifluoroethanol (benzyl alcohol) would be expected to be present in the alcoholate ion form at pH 5.7. Substitution of trifluoroethanol by benzyl alcohol in the



Scheme 4. Proposed reaction mechanism for proton transfer and (benzyl) alcohol binding during liver alcohol dehydrogenase catalysis

ternary enzyme · NAD⁺ · alcohol complex, therefore, should result in an uptake of about 0.8 active-site-equivalent of proton at this pH. The results in Fig. 5 establish that such an uptake of close to one equivalent of proton does take place on displacement of trifluoroethanol with benzyl alcohol at pH 5.7. It may be noted that the latter observation cannot be reconciled with the binding mechanism proposed by Shore et al. [2]; Scheme 1 predicts that no proton transfer to or from solution will occur on substitution of one alcohol by another in the ternary enzyme · NAD⁺ · alcohol complex.

The present investigation provides evidence that enzymic hydride transfer from benzyl alcohol to NAD⁺ and trifluoroethanol binding to the enzyme · NAD⁺ complex conform to the reaction mechanism shown in Scheme 2 and 3, respectively. It seems reasonable to assume that the binding pattern established for trifluoroethanol is valid also for alcohol substrates. The present, results, therefore, suggest that alcohol binding and proton transfer during the catalytic action of liver alcohol dehydrogenase proceed by the mechanism indicated in Scheme 4. Direct evidence in favour of this reaction scheme is presented in the following paper, where the mechanistic implications of the present results are discussed in more detail.

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Unified Mechanism for Proton-Transfer Reactions Affecting the Catalytic Activity of Liver Alcohol Dehydrogenase

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The effect of pH on substrate binding to liver alcohol dehydrogenase has been examined over the pH range 6–10 by transient-state and steady-state kinetic methods. The results provide evidence that there is no significant effect of pH on benzaldehyde binding to the enzyme. Benzyl alcohol association to the binary enzyme · NAD⁺ complex requires protonation of an ionizing group with a pK_a of 7.6 in the binary complex. Substrate dissociation from the enzyme · NAD⁺ · alcohol complex is regulated by an ionizing group with a pK_a of 6.6 (6.4) in the complex formed with naphthyl alcohol (benzyl alcohol). Alcohol desorption from the ternary complex occurs exclusively when the ionizing group is in the protonated form.

A reaction mechanism is proposed which accounts for all major effects of pH on liver alcohol dehydrogenase catalysis over the investigated pH range. The reactivity of the enzyme · NAD⁺ (enzyme · NAD⁺ · alcohol) complex is suggested to be regulated by the ionization state of a water (alcohol) molecule bound at the catalytic zinc atom of the enzyme. Zinc-bound water does not function as a binding site for substrates or as a mediator of catalytic proton transfer from substrate to solution at the binary-complex level. Catalytic proton transfer takes place at the ternary-complex level, probably through an alcohol/alcoholate ion interconversion of the enzyme-bound substrate. This proton transfer step can be envisaged to serve the purpose of facilitating hydride transfer during alcohol oxidation and alcohol desorption during aldehyde reduction. The kinetics of proton uptake/release during naphthaldehyde reduction at pH 6 are shown to be consistent with the proposed mechanism of enzyme action.

Liver alcohol dehydrogenase catalyzes the oxidation of various alcohols by NAD⁺ and the reverse reaction of aldehyde reduction by NADH [1]. The enzyme operates by a ternary-complex mechanism which, in general, can be assumed to be effectively ordered with coenzyme binding preceding substrate binding and product release preceding coenzyme release [2]. The corresponding kinetic scheme is usually written as in Scheme 1 and has been shown to account for the steady-state [2] and transient-state [3–5] rate behaviour of the enzyme under conditions where no substrate inhibition or substrate activation is observed.

Aldehyde formation during the catalytic action of the enzyme requires a net removal of two hydrogen atoms from the alcohol substrate. This dehydrogenation process is known to proceed by a mechanism of combined proton and hydride ion transfer, and it has been well established that transfer of the hydride ion

occurs directly between substrate and coenzyme in the productive ternary complex formed during catalysis [1]. The mechanism of proton transfer has been less well characterized, due partly to the fact that protons have a dual effect on the catalytic activity of the enzyme. Besides participating in the catalytic reaction as a substrate or product, the proton acts as a modifier of enzyme activity through regulation of the ionization state of catalytically essential groups which may, or may not, function as mediators of proton transfer from substrate to solution. A detailed understanding of the enzymic dehydrogenation process, therefore, requires characterization and a coherent description of all proton transfer steps which affect the catalytic activity of the enzyme.

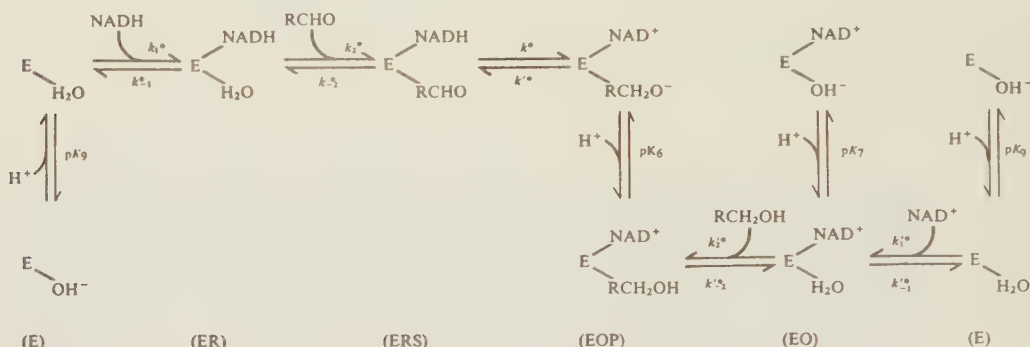
To arrive at such a characterization of the catalytic reaction mechanism, we have carried out systematic studies of the effect of pH on individual reaction steps in Scheme 1. Previously reported results of these studies indicate that three different proton transfer steps have to be considered to account for the observed pH de-

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).



Scheme 1. The ordered ternary-complex mechanism usually considered for evaluation of the kinetics of liver alcohol dehydrogenase catalysis

E, R, and O denote enzyme, NADH, and NAD⁺, respectively. S stands for an aldehyde substrate and P for the corresponding alcohol



Scheme 2. Proton equilibria suggested to affect the catalytic activity of liver alcohol dehydrogenase

Ionizing groups on the enzyme are tentatively identified as a water or alcohol molecule bound at the catalytic zinc atom of the enzyme

pendence of coenzyme binding [6] and ternary-complex interconversion [5]. The results described in the preceding paper suggest that these proton transfer steps are linked to the catalytic sequence of reactions as indicated by the extended reaction mechanism in Scheme 2. In the present investigation we show that Scheme 2 is sufficient to account also for the observed pH dependence of substrate binding to the enzyme.

This means that our series of pH dependence studies of the liver alcohol dehydrogenase system has led to a coherent mechanistic description of all main effects of pH on the enzymic reaction over the investigated pH range (6–10). The characteristic features of Scheme 2, therefore, are discussed in considerable detail with particular reference to the mechanism of substrate binding and proton transfer during catalysis.

EXPERIMENTAL PROCEDURE

Unless otherwise stated, Materials and Methods used in the present investigation were the same as detailed in the preceding paper [8]. The substrates benzyl alcohol and benzaldehyde (BDH Chemicals Ltd) were distilled at reduced pressure under nitrogen immediately before use. β -Naphthaldehyde (Merck AG) was purified by sublimation in vacuum.

Kinetic measurements were performed at 25 °C in 0.1 M ionic strength phosphate (pH 6–8), pyrophosphate (pH 8–9), or carbonate (pH 9–10) buffer. Steady-state kinetic coefficients for the enzymic oxida-

tion of benzyl alcohol and reduction of benzaldehyde were determined by standard spectrometric methods [7] using a Beckman Acta CIII spectrophotometer.

The transient-state kinetics of aldehyde reduction were examined by stop-flow techniques as described previously [3]. Reaction solutions contained about 10 μ M enzyme (active-site concentration), 10 mM pyrazole, a saturating concentration of NADH (usually 0.2 mM), and varied non-saturating concentrations of benzaldehyde (0.1–5 mM) or naphthaldehyde (20–200 μ M). Reactions were monitored at 297 nm after mixing the enzyme from one syringe with other reactants from the second syringe. The apparent first-order rate constant (r_1) for the single-exponential transient observed at 297 nm was determined by regression analysis [5]. The limiting value ($r_{1,\infty}$) of r_1 at saturating substrate concentrations was estimated by the extrapolation procedure described previously [3].

Proton uptake/release measurements in the reaction between β -naphthaldehyde and the enzyme $\cdot$ NADH complex were made by stop-flow techniques at pH 6.0 in 33 mM sodium sulphate solutions buffered with 20 μ M chlorophenol red. One syringe contained enzyme (30 μ M), preincubated with 150 μ M NADH. The second syringe contained 60 μ M β -naphthaldehyde and 20 mM trifluoroethanol. This concentration of trifluoroethanol is sufficiently high to obtain a rapid [8] and virtually stoichiometric [9] conversion of the catalytically produced enzyme $\cdot$ NAD⁺ complex into the non-productive enzyme $\cdot$ NAD⁺ $\cdot$ trifluoroethanol complex.

RESULTS

Effect of pH on Alcohol Dissociation Rates

The saturation value $r_{1,\infty}$ of the rate parameter for the slowest exponential transient governing the kinetics of aldehyde reduction by liver alcohol dehydrogenase under single-turnover conditions [3] has been shown to be related to velocity constants in Scheme 1 [10] through Eqn (1):

$$r_{1,\infty} = \frac{k k'_{-2}}{k + k' + k'_{-2}} \quad (1)$$

Fig. 1 shows the effect of pH on $r_{1,\infty}$ for the enzymic reduction of β -naphthaldehyde between pH 5.5 and 9. With this substrate we have $k' \ll k \approx 400 \text{ s}^{-1}$ over the investigated pH range [11]. This means that the rate constant k'_{-2} for alcohol dissociation from the EOP species in Scheme 1 provides a major contribution limiting the magnitude of $r_{1,\infty}$. The data in Fig. 1, therefore, confirm the previous conclusion that the rate of naphthyl alcohol dissociation decreases strongly with increasing pH [11].

More precise estimates of k'_{-2} for naphthyl alcohol dissociation were calculated from the experimental observations of $r_{1,\infty}$ using Eqn (1) with the assumption that $k' \ll k = 400 \text{ s}^{-1}$ [11]. The k'_{-2} values thus obtained at different pH are shown in Fig. 2. As would be expected from the results described in the preceding paper, experimental points in Fig. 2 conform well to a proton dissociation function of the form

$$k'_{-2} = \frac{k'^*_{-2}}{1 + \frac{K_6}{[\text{H}^+]}} \quad (2)$$

The best-fit parameter estimates obtained by regression analysis of the data in Fig. 2 were $k'^*_{-2} = 140 (\pm 20) \text{ s}^{-1}$ and $\text{p}K_6 = 6.6 (\pm 0.2)$. The latter estimate of the $\text{p}K_a$ regulating naphthyl alcohol dissociation from the enzyme $\cdot \text{NAD}^+$ alcohol complex does not differ significantly from the $\text{p}K_a$ of 6.4 reported to regulate the catalytic activity of the corresponding ternary complex formed benzyl alcohol [5] and ethanol [12].

The parameter $r_{1,\infty}$ for the enzymic reduction of benzaldehyde was similarly determined at different pH between 7 and 10 (Fig. 1). The corresponding values of k'_{-2} , calculated from Eqn (1) with the assumption that $k' \ll k = 290 \text{ s}^{-1}$ [3,5], are shown in Fig. 3 and can be seen to be approximately proportional to the hydrogen ion concentration over the investigated pH range. This observation lends additional support to the idea that alcohol dissociation rates are regulated by the relationship in Eqn (2). Although the data in Fig. 3 cannot be used for estimation of the equilibrium constant K_6 in Eqn (2), they do establish that $\text{p}K_6$ must be lower than 7. Using the previously reported value $\text{p}K_6 = 6.4$ for benzyl alcohol/

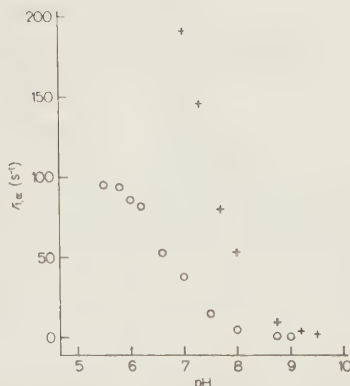


Fig. 1. Effect of pH on the rate parameter for the slowest exponential transient governing the kinetics of aldehyde reduction in the liver alcohol dehydrogenase system. Saturation values $r_{1,\infty}$ determined by extrapolation to infinite substrate concentration of apparent first-order rate constants for the transient observed at 297 nm on reacting the enzyme (about $10 \mu\text{M}$) with varied concentrations of β -naphthaldehyde (O) or benzaldehyde (+) at 25°C in 0.1 M ionic strength buffer solutions containing 0.2 mM NADH and 10 mM pyrazole

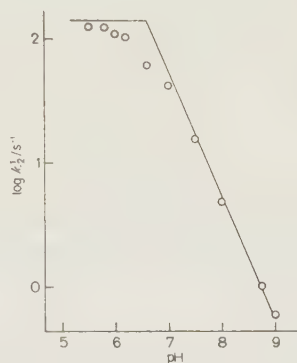


Fig. 2. Effect of pH on the rate constant for naphthyl alcohol dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Estimates of k'_{-2} calculated from the data in Fig. 1 using Eqn (1) with the assumption that $k' \ll k = 400 \text{ s}^{-1}$. Experimental points conform to a proton dissociation function with a $\text{p}K_a$ of 6.6

benzaldehyde interconversion [5], a fit of Eqn (2) to the data in Fig. 3 gave $k'^*_{-2} = 2500 (\pm 400) \text{ s}^{-1}$.

The pH dependence of the rate constant k' in Scheme 1 has been shown to be given by

$$k' = \frac{k'^*}{1 + \frac{K_6}{[\text{H}^+]}} \quad (3)$$

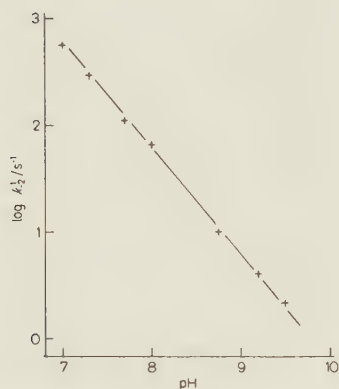


Fig. 3. Effect of pH on the rate constant for benzyl alcohol dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Estimates of k'_{-2} calculated from the data in Fig. 1 using Eqn (1) with the assumption that $k' \ll k = 290 \text{ s}^{-1}$

with $k'^* = 18 \text{ s}^{-1}$ and $\text{p}K_6 = 6.4$ for the enzymic interconversion of benzyl alcohol and benzaldehyde [5]. Assuming that k'_{-2} for benzyl alcohol dissociation conforms to Eqn (2) with $\text{p}K_6 = 6.4$ and $k'^*_{-2} = 2500 \text{ s}^{-1}$, it follows that the pH dependence of the quotient k'/k'_{-2} can be expressed as

$$\frac{k'}{k'_{-2}} = \frac{K_8}{[\text{H}^+]} \quad (4)$$

where

$$K_8 = \frac{k'^* K_6}{k'_{-2}} \quad (5)$$

corresponding to $\text{p}K_8 = 8.5$ for benzyl alcohol/benzaldehyde interconversion. This prediction will be tested in the following sections.

Effect of pH on Aldehyde Binding

The steady-state kinetic coefficient ϕ_2 for the enzymic reduction of aldehydes is related to velocity constants in Scheme 1 [7] through

$$\phi_2 = \frac{1}{k_2} \left[1 + \frac{k_{-2}}{k} \left(1 + \frac{k'}{k'_{-2}} \right) \right] \quad (6)$$

When benzaldehyde is used as the substrate, we may assume that $k_{-2} \gg k$ over the pH range 6–10 [5] and Eqn (6) reduces to

$$\phi_2 = \frac{k_{-2}}{k_2 k} \left(1 + \frac{k'}{k'_{-2}} \right) \quad (7)$$

Insertion of Eqn (4) then gives

$$\phi_2 = \frac{k_{-2}}{k_2 k} \left(1 + \frac{K_8}{[\text{H}^+]} \right) \quad (8)$$

with $\text{p}K_8 = 8.5$.

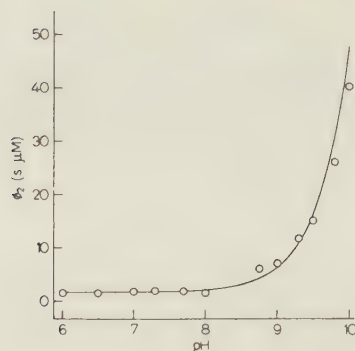


Fig. 4. Effect of pH on the steady-state kinetic coefficient ϕ_2 for benzaldehyde reduction in the liver alcohol dehydrogenase system. The curve drawn was calculated from Eqn (8) with the assumption that $k_{-2}/k_2 k = 1.6 \mu\text{M s}$ and $\text{p}K_8 = 8.5$

To test the validity of Eqn (8), estimates of ϕ_2 for the enzymic reduction of benzaldehyde were determined at different pH between 6 and 10 by conventional steady-state kinetic methods. The results are shown in Fig. 4 and provide clear evidence that ϕ_2 exhibits the expected dependence on a proton dissociation step with an apparent $\text{p}K_a$ close to 8.5. This observation confirms the validity of Eqns (4) and (5) and hence the validity of the assumption that k'_{-2} for benzyl alcohol dissociation conforms to Eqn (2) with $\text{p}K_6 = 6.4$.

The data in Fig. 4 provide the additional information that the pH dependence of ϕ_2 is fully accounted for by a single monobasic dissociation function. According to Eqn (8), this means that the magnitude of the quotient $k_{-2}/k_2 k$ remains essentially constant between pH 6 and 10. Since the magnitude of k is known to be independent of pH over this range [5, 11], the data in Fig. 4 provide direct evidence that there is no significant effect of pH on the equilibrium constant k_{-2}/k_2 for benzaldehyde binding to the enzyme · NADH complex.

A fit of Eqn (7) to the data in Fig. 4 gave $\text{p}K_8 = 8.5 (\pm 0.2)$ and $k_{-2}/k_2 k = 1.6 (\pm 0.2) \mu\text{M s}$. Assuming that $k = 290 \text{ s}^{-1}$ for benzaldehyde reduction [3, 5], it follows that $k_{-2}/k_2 = 460 (\pm 60) \mu\text{M}$. The latter estimate of the equilibrium constant for benzaldehyde dissociation from the productive ternary complex agrees well with that obtained previously by transient-state kinetic techniques ($k_{-2}/k_2 \approx (k + k_{-2})/k_2 = 400 \mu\text{M}$ [3]). This appears to establish the validity of the present interpretation of the observed pH dependence of ϕ_2 .

Eqns (4–8) show that the pH dependence of ϕ_2 for benzaldehyde reduction derives from the effect of

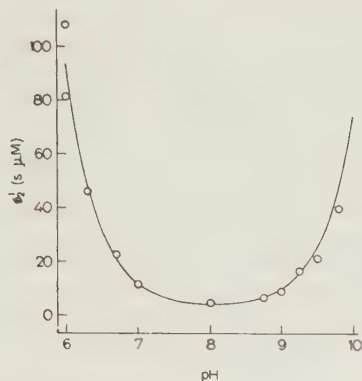


Fig. 5. Effect of pH on the steady-state kinetic coefficient ϕ_2' for benzyl alcohol oxidation in the liver alcohol dehydrogenase system. The curve drawn was calculated from Eqns (10) and (11) with the assumption that $pK_7 = 7.6$, $pK_8 = 8.5$, and $k_2'^* = 3.5 \text{ s}^{-1} \mu\text{M}^{-1}$.

the pK_6 proton equilibrium (Scheme 2) on the relative magnitude of k' and k_{-2}' . At low pH, alcohol production is rate-limited by the hydride transfer step ($k' \ll k_{-2}'$), whereas alcohol desorption becomes rate-limiting at high pH ($k_{-2}' \ll k'$). The variation with pH of isotope effects on the rate of reduction of the quasi-substrate *trans-N,N*-dimethylaminocinnamaldehyde has been similarly interpreted in terms of a proton-dependent shift of the rate-limiting step [13].

Effect of pH on Alcohol Association Rates

The steady-state kinetic coefficient ϕ_2' for enzymic alcohol oxidation is related to rate constants in Scheme 1 [7] through

$$\phi_2' = \frac{1}{k_2'} \left[1 + \frac{k_{-2}'}{k_2'^*} \left(1 + \frac{k}{k_{-2}} \right) \right]. \quad (9)$$

It follows from this relationship and Eqn (4) that

$$\phi_2' = \frac{1}{k_2'} \left(1 + \frac{[\text{H}^+]}{K_8} \right) \quad (10)$$

when benzyl alcohol is used as the substrate ($k_{-2} \gg k$). Eqn (10) shows that examination of the effect of pH on ϕ_2' for benzyl alcohol oxidation will provide comparatively direct information about the magnitude and pH dependence of the rate constant k_2' for alcohol association to the enzyme $\cdot \text{NAD}^+$ complex.

Steady-state kinetic estimates of ϕ_2' for the enzymic oxidation of benzyl alcohol were determined over the pH range 6–10. As indicated by the results in Fig. 5, ϕ_2' exhibits an obvious dependence on two proton dissociation equilibria with an opposite effect on the magnitude of ϕ_2' . According to Eqn (10), this observation provides evidence that the magnitude of k_2' varies with

pH and suggests that the pH dependence of k_2' can be expressed in terms of a monobasic dissociation function of the form

$$k_2' = \frac{k_2'^*}{1 + \frac{K_7}{[\text{H}^+]}}. \quad (11)$$

Regression analysis established that Eqns (10) and (11) can be well fitted to the data in Fig. 5 using the pK_8 value of 8.5 prescribed by Eqn (5). The best-fit estimate of pK_7 thus obtained (7.7) did not differ significantly from the pK_8 value (7.6) previously reported to regulate the binding properties of the enzyme $\cdot \text{NAD}^+$ complex [6,9]. Assuming that $pK_7 = 7.6$ and $pK_8 = 8.5$, a fit of Eqns (10) and (11) to the data in Fig. 5 gave $k_2'^* = 3.5 (\pm 0.6) \text{ s}^{-1} \mu\text{M}^{-1}$.

Proton Transfer at the Ternary-Complex Level

In the preceding paper [8] we showed that hydride transfer during the enzymic oxidation of benzyl alcohol is coupled to a kinetically detectable release of protons. To demonstrate that there is a corresponding uptake of protons at the ternary-complex level during aldehyde reduction, the enzyme $\cdot \text{NADH}$ complex was reacted with β -naphthaldehyde at pH 6.0 in a solution buffered with the pH indicator chlorophenol red. The reaction was carried out under pseudo-single-turnover conditions by 'catalytic suicide' methods [14], using trifluoroethanol to trap the catalytically produced enzyme $\cdot \text{NAD}^+$ complex.

According to previous reports [11,14], transient absorbance changes due to NADH consumption in this reaction would be expected to exhibit a biphasic time course. The lower trace in Fig. 6, which shows the absorbance changes recorded at 320 nm, confirms that such is the case. A rapid (350 s^{-1}) exponential transient, known to reflect the hydride transfer step, is followed by a slower (65 s^{-1}) transient which reflects alcohol desorption and subsequent rapid formation of the trapped enzyme $\cdot \text{NAD}^+$ complex [10,11,14]. The upper trace in Fig. 6 shows that 574-nm absorbance changes (due to minor changes in pH) during the reaction are governed by two exponential transients exhibiting the same rates as those observed at 320 nm. There is an initial rapid uptake of protons (occurring partly during the 2.0-ms dead time of the stop-flow apparatus) in the reaction phase reflecting hydride transfer, and a subsequent slower release of approximately the same amount of protons in the transient phase reflecting alcohol desorption. Amplitudes of the two transients correspond to an uptake and subsequent release of $0.5 (\pm 0.1)$ proton/active site of the enzyme.

The results in Fig. 6 can be readily explained in view of Scheme 2. Formation of the enzyme $\cdot \text{NADH}$ $\cdot$ naphthaldehyde complex, which can be assumed to

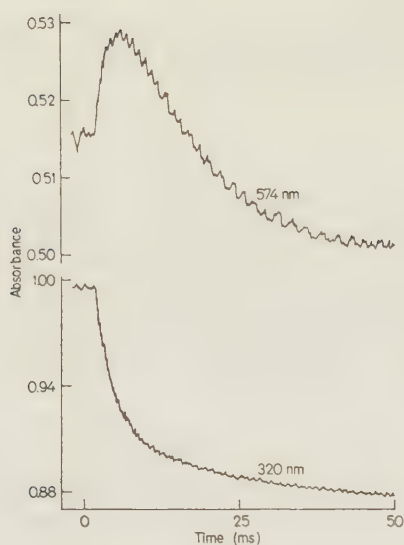


Fig. 6. *NADH* consumption and proton uptake/release in the reaction between naphthaldehyde and the binary enzyme · *NADH* complex. Absorbance changes recorded at 320 nm (lower trace) and 574 nm (upper trace) after mixing 30 μ M liver alcohol dehydrogenase, pre-incubated with 150 μ M *NADH*, with 60 μ M β -naphthaldehyde and 20 mM trifluoroethanol at 25 °C in 33 mM sodium sulphate solution buffered with 20 μ M chlorophenol red

be completed within the dead time of the stop-flow apparatus, is followed by hydride transfer leading to formation of the enzyme · NAD^+ · naphthylalcoholate ion complex. Since the latter complex rapidly equilibrates with the neutral enzyme · NAD^+ · naphthyl alcohol complex ($pK_6 = 6.6$), hydride transfer at pH 6.0 will result in an uptake of 0.80 proton/*NADH* molecule formed. Subsequent naphthyl alcohol desorption and rapid trifluoroethanol association to the enzyme · NAD^+ complex leads to liberation of the protons taken up in the hydride transfer step because the ternary complex formed with trifluoroethanol ($pK_6 = 4.3$ [8]) can be assumed to be fully ionized at pH 6.0.

This means that proton uptake/release during the reaction would be expected to reflect mainly formation and breakdown of the species EOP in Scheme 1, the amplitude of the transients governing proton uptake/release being 80% of that of the transient describing the time dependence of the concentration of EOP. The latter amplitude has been shown [10] to be given approximately by the quotient between the rate parameter for the slow transient (65 s^{-1}) and the rate constant k'_{-2} for alcohol dissociation from the ternary complex (110 s^{-1} according to the data in Fig. 2). The amplitude of the transient uptake and release of pro-

tons, therefore, would be expected to equal $0.8 \times 65 / 110 = 0.47$ active-site equivalent, which is in excellent agreement with the experimentally observed value. It may be concluded that the results in Fig. 6 are qualitatively and quantitatively consistent with the proton transfer mechanism in Scheme 2.

It follows from the above interpretation of the data in Fig. 6 that transient proton uptake due to formation of an enzyme · NAD^+ · alcohol complex can be anticipated to exhibit a low amplitude at alkaline pH where the ternary complex will be mainly in the unprotonated form. This explains why proton uptake at the ternary-complex level could not be detected by Dunn in his kinetic studies of azoaldehyde reduction at pH 8.75 [15].

DISCUSSION

Descriptive Validity of Scheme 2

Kinetic data for the liver alcohol dehydrogenase system are usually evaluated in view of the compulsory-order mechanism in Scheme 1. Assuming that proton transfer steps equilibrate rapidly in comparison to other reaction steps, relationships between rate constants in Scheme 1 and those defined by the extended mechanism in Scheme 2 can be derived by elementary equilibrium analysis. Such an analysis shows that the pH dependence predicted by Scheme 2 for rate constants in Scheme 1 can be expressed in terms of three different proton dissociation functions of the form

$$f(K_i) = \left(1 + \frac{K_i}{[\text{H}^+]}\right)^{-1}; \quad i = 6, 7, 9 \quad (12)$$

as indicated in Table 1.

Our previous studies of the effect of pH on liver alcohol dehydrogenase catalysis have established that the pH dependence of coenzyme binding [6] and ternary-complex interconversion [5] conforms well to the relationships in Table 1 over the investigated pH range (6–10). The present investigation, therefore, has been directed towards a characterization of the effect of pH on substrate binding to the enzyme. Such a characterization has to be made by kinetic methods; the catalytically relevant step of substrate binding leads to formation of a productive ternary complex and cannot be examined by equilibrium methods. The present paper describes the effect of pH on three kinetic parameters (ϕ_2 , ϕ'_2 , $r_{1,\infty}$) which are known to be more or less directly related to the process of substrate binding.

The results provide clear evidence that the pH dependence of benzyl alcohol and benzaldehyde binding conforms to the relationships in Table 1. This means that Scheme 2 (with $pK_6 = 6.4$, $pK_7 = 7.6$, and $pK_9 = 9.2$ [5, 6]) provides a coherent mechanistic ex-

Table 1. Effect of pH on rate constants in Scheme 1 according to the proton-transfer mechanism in Scheme 2

The proton dissociation function $f(K_i)$ is defined in Eqn (12)

Rate constant in Scheme 1	Kinetic equivalent in terms of constants in Scheme 2
k_1	$k_1^* f(K_a)$
k_{-1}	k_{-1}^*
k_2	k_2^*
k_{-2}	k_{-2}^*
k	k^*
k_1'	$k_1^* f(K_b)$
k_{-1}'	$k_{-1}^* f(K_7)$
k_2'	$k_2^* f(K_5)$
k_{-2}'	$k_{-2}^* f(K_6)$
k'	$k^* f(K_6) K_b [H^+]$

Table 2. Estimates of rate and equilibrium constants in Scheme 2 for the enzymic interconversion of benzyl alcohol and benzaldehyde

Constant	Unit	Estimate	Reference
k_1^*	$s^{-1} \mu M^{-1}$	12	[6]
k_{-1}^*	s^{-1}	6	[6]
k_{-2}^*/k_2^*	μM	460	present work
k^*	s^{-1}	290	[5]
$k_1'^*$	$s^{-1} \mu M^{-1}$	1.0	[6]
$k_{-1}'^*$	s^{-1}	160	[6]
$k_2'^*$	$s^{-1} \mu M^{-1}$	3.5	present work
$k_{-2}'^*$	s^{-1}	2500	present work
k'^*	s^{-1}	18	[5]
pK_6		6.4	[5]
pK_7		7.6	[6]
pK_9		9.2	[6]

planation for the effect of pH on each individual rate constant in Scheme 1 with these substrates. Our estimates of the corresponding pH-independent rate constants in Scheme 2 are listed in Table 2. These estimates correspond to an equilibrium constant of 5×10^{-11} M for the overall reaction, which agrees well with the values determined previously by more direct methods [4, 14]. Using the data in Tables 1 and 2, magnitudes of rate constants in Scheme 1 can be calculated for benzyl alcohol/benzaldehyde interconversion at any pH between 6 and 10. The values thus obtained for the reaction at pH 8.75 agree satisfactorily with those reported by Weidig et al. [4]. These observations lend credence to the present analysis and interpretation of the kinetic parameters examined.

Dunn et al. [16] have reported that on-velocity and off-velocity constants for binding of the chromophoric quasi-substrate *trans*-*N,N*-dimethylaminocinnamaldehyde to the enzyme $\cdot$ NADH complex are independent of pH over the range 6–10.5. This is consistent with Scheme 2 and the present conclusion that there is no effect of pH on the binding of a more

typical substrate such as benzaldehyde. The latter conclusion was based on the observation that the parameter ϕ_2 for benzaldehyde reduction conforms to a monobasic dissociation function, approaching a constant value at low pH. According to Eqn (6) and the relationships in Table 1, ϕ_2 would be expected to exhibit a similar pH profile with other substrates (except that the apparent pK_a regulating the magnitude of ϕ_2 may differ from 8.5). Reported steady-state kinetic data for acetaldehyde reduction indicate that such is the case [17, 18]. The lack of effect of pH on aldehyde binding, therefore, appears to be a general characteristic of liver alcohol dehydrogenase catalysis.

It can be similarly inferred from the reported effect of pH on ϕ_2' for ethanol oxidation [17, 18] that association of the latter substrate to the enzyme $\cdot$ NAD $^+$ complex most likely exhibits a pH dependence analogous to that observed for benzyl alcohol association. Furthermore, the data in Fig. 2 and 3 establish that Eqn (2) applies to both naphthyl alcohol and benzyl alcohol dissociation. Additional evidence that the kinetics of alcohol binding are generally consistent with the relationships in Table 1 was presented in the preceding paper. For these reasons, there seems to be little doubt that Scheme 2 accounts for the pH dependence of liver alcohol dehydrogenase catalysis independently of the substrate being acted upon.

The present investigation appears to establish that three proton equilibria, linked to the catalytic sequence of reactions as indicated in Scheme 2, have to be considered to explain the main effects of pH on liver alcohol dehydrogenase catalysis over the investigated pH range. The kinetic data presented do not, however, provide any direct information about the identity of the ionizing groups which participate in these proton equilibria. Arguments favouring the suggestion in Scheme 2 that the proton dissociation reactions involve an enzyme-bound water or alcohol molecule will be considered in the following discussion.

Mechanism of Substrate Binding

Crystallographic data presented by Brändén and coworkers [1] indicate that the substrate binding site in liver alcohol dehydrogenase consists of a deep hydrophobic pocket with a hydrophilic bottom where the catalytic reaction occurs. The hydrophilic bottom contains the catalytic zinc atom of the enzyme, coordinated to three protein ligands. A tetragonal coordination to the metal is completed by a water molecule (or hydroxyl ion) projecting into the substrate binding pocket. The nicotinamide portion of the co-enzyme is bound near the catalytic zinc atom, suggesting that substrates coordinate to zinc-bound water on ternary-complex formation. Kinetic [19], spectroscopic [20], and structural [21] evidence in favour of this idea has been presented.

Shore et al. [9] have proposed a mechanism for the catalytic action of the enzyme, according to which the binding of NAD^+ perturbs the pK_a of an ionizing group from about 9 to 7.6 with concomitant proton release corresponding to the proton liberated in the overall reaction. Alcohol substrates were envisaged to combine to the unprotonated form of the enzyme $\cdot \text{NAD}^+$ complex through direct interaction with the basic form of the pK_a -7.6 group. A modification of the mechanism in the latter respect was proposed by Brändén et al. [1], who identified the pK_a -7.6 group as zinc-bound water and suggested that the substrate is bound directly to zinc as an alcoholate ion. Formation of the alcoholate ion was assumed to be mediated by the zinc-bound hydroxyl ion and to result in displacement of this ligand as a water molecule. More recently, Dworschack et al. [22] advanced the idea that the neutral alcohol adds as a fifth ligand to zinc while the hydroxyl ion remains zinc-bound and functions as a proton acceptor in the subsequent dehydrogenation step.

A common characteristic of the above mechanistic proposals is the assumption that alcohol substrates combine exclusively to the unprotonated form of the enzyme $\cdot \text{NAD}^+$ complex. The validity of this assumption was questioned in the preceding paper [8], where it was shown that ternary-complex formation with the substrate analogue trifluoroethanol does not proceed by such a mechanism. The present investigation establishes that the binding of alcohol substrates conforms to the same kinetic scheme as does trifluoroethanol binding, alcohol ligands combining preferentially to the protonated form of the enzyme $\cdot \text{NAD}^+$ complex (Scheme 2). This observation rules out the mechanisms of enzyme action proposed by Shore et al. [9], Brändén et al. [1] and Dworschack et al. [22], as well as any alternative mechanism which attributes a catalytically essential alcohol-binding function to the unprotonated form of the binary complex.

To our opinion [6], it has been well established that the pK_a -7.6 dependence of NAD^+ and alcohol binding derives from ionization of zinc-bound water [1]. Assuming that such is the case, it may be concluded from the present results that alcohol association to the enzyme $\cdot \text{NAD}^+$ complex requires the presence of a neutral water molecule at the catalytic zinc atom. This observation precludes that a zinc-bound hydroxyl ion in the binary functions as an immediate site for alcohol substrates or acts as a base-catalyst for direct association of the substrate in the form of an alcoholate ion. The possibility that alcohols bind in the second coordination sphere through hydrogen bonding to zinc-bound water is also rendered highly unlikely; ionization of the water molecule would not be expected to prevent the formation of such an outer-sphere complex. The present results, therefore, lend strong support to the idea that alcohol and, by inference,

aldehyde substrates coordinate directly to zinc on ternary-complex formation.

It may prove difficult to establish whether or not such a coordination of the substrate to zinc results in formation of a penta-coordinate complex. Considering the evidence presented up to date, however, there seems to be no strong argument favouring the proposal that substrate binding takes place without displacement of (tetra- or possibly penta-coordinate) water. The kinetic data reported by Dworschack et al. [22] can be readily understood without the assumption that water remains zinc-bound in the productive ternary complexes. The limiting rates observed by Frolich et al. [23] for the association of bidentate zinc-chelating agents seem to be far too low to represent water dissociation rates [24–26] and more likely reflect the multistage character of the process of chelate formation. Moreover, it may be questioned if coordination of the substrate to zinc without displacement of water is consistent with the kinetic data reported in the present investigation.

However that may be, the simplest and most reasonable interpretation of the present results is that substrate binding takes place with displacement of water through a dissociative mechanism [25] of ligand substitution. According to this idea, ionization of zinc-bound water prevents its substitution due to the tighter binding and/or slower rate of dissociation of the negatively charged hydroxyl ion. This explains the observed pK_a -7.6 dependence of decanoate [8] and alcohol association to the enzyme $\cdot \text{NAD}^+$ complex, as well as the lack of effect of pH on aldehyde binding to the enzyme $\cdot \text{NADH}$ complex over the investigated pH range; as has been pointed out previously [6,18], the pK_a of zinc-bound water must be assumed to be higher than 10 in the latter binary complex. Furthermore, a reasonable explanation is provided for the observation that alcohol association rates (even the maximum rates observed at low pH) are considerably lower than expected for a diffusion-controlled reaction. Factors other than diffusion are known to control and limit the rate of a substitution process [25].

The present results establish that the ternary complex formed with alcohol substrates and NAD^+ participates in a proton-transfer step which regulates the rate of aldehyde formation and alcohol dissociation from the ternary complex (the pK_6 step in Scheme 2). This observation can be well understood in view of a substitution mechanism for substrate binding. By analogy to zinc-bound water, the zinc-bound alcohol would be expected to exhibit a considerably enhanced tendency to ionize (with alcoholate ion formation) when NAD^+ is bound to the enzyme. We have suggested previously that the pK_a -6.4 dependence of the apparent rate of hydride transfer to NAD^+ from ethanol or benzyl alcohol can be attributed to such a step of rapid alcohol/alcoholate ion interconversion of the enzyme-

bound substrate [5], and additional evidence in support of this suggestion was reported in the preceding paper [8]. The observed pH dependence of alcohol dissociation from the ternary complex can be explained as an effect of the same ionization process displacement of the neutral alcohol molecule occurring at a considerably higher rate than displacement of the alcoholate ion. Direct evidence that the alcohol ligand forms part of the ionizing structure which regulates the reactivity of the ternary complex is provided by the observation that the corresponding pK_a value is dependent on the electronic properties of the ligand (pK_a 6.4 with ethanol [12]; pK_a 4.3 with trifluoroethanol [8]).

The idea that the substrate is bound directly to zinc as an alcoholate ion in the productive ternary complex is mechanistically sound and has been previously advanced by several authors [1,27,28]. The present results indicate a realistic pathway (Scheme 2) for the formation of such a complex. It follows from Scheme 2 that the apparent affinity of the enzyme $\cdot NAD^+$ complex for alcohols is dependent on three different factors: the destabilizing effect of ionization of zinc-bound water in the binary complex, the stabilizing effect of alcoholate ion formation in the ternary complex, and the equilibrium constant for the actual dissociation of the neutral alcohol from the ternary complex. In the case of benzyl alcohol binding, the data in Table 1 show that the latter equilibrium constant ($k_{-2}^*/k_2^* = 0.8$ mM) agrees well in magnitude with the equilibrium constant for benzaldehyde dissociation from the enzyme $\cdot NADH$ $\cdot$ aldehyde complex ($k_{-2}^*/k_2^* = 0.5$ mM). Several inferences can be drawn from this observation. Firstly, it appears that interactions of similar strength contribute to the binding of the aldehyde and the corresponding neutral alcohol. This is what might be expected for a substitution mechanism, since the aldehyde and the neutral alcohol are likely to form structurally similar ternary complexes stabilized mainly through hydrophobic interactions and coordination to zinc. Secondly, it follows that the oxidation-reduction state of the coenzyme has no drastic effect on the stability of the complexes formed with the uncharged substrates. Conformational changes induced by the binding of $NADH$ and NAD^+ [1] either have a similar effect or no effect at all on the substrate-binding capacity of the enzyme. Finally, it may be concluded that the apparent tighter binding of benzyl alcohol relatively to benzaldehyde (which is most pronounced at alkaline pH) can be attributed entirely to the stabilization achieved by alcoholate ion formation at the ternary-complex level.

According to this interpretation, the pH dependence of alcohol binding to the enzyme $\cdot NAD^+$ complex derives from two analogous ionization processes involving, respectively, zinc-bound water (pK_a 7.6) and the zinc-bound alcohol (pK_a 6.4 with ethanol and

benzyl alcohol). This means that both water and alcohol ligands must be assumed to become drastically more acid on binding to the enzyme $\cdot NAD^+$ complex, the pK_a perturbation being considerably larger for alcohols than for water.

Several factors may contribute to these pK_a perturbations. Coordination to zinc (and possibility hydrogen bonding to the proton relay system mentioned below) is likely to provide a major contribution, corresponding to a pK_a shift of about 5–7. This can be inferred from the pK_a value of zinc-bound water in free enzyme, which is generally considered to be close to 9 [1] and certainly would be expected not to exceed 11. The additional decrease in pK_a caused by NAD^+ binding can be attributed to electrostatic interaction between the ionized zinc-bound ligand and the positively charged nicotinamide ring of the coenzyme [18]. Considering the short distance between the two charges and their location in a hydrophobic environment [1], it seems quite reasonable that such an interaction may stabilize the basic form of the ligand by up to 20–35 kJ mol⁻¹, corresponding to an additional pK_a shift of 3–6. The electrostatic pK_a perturbation would be expected to be considerably larger for a zinc-bound alcohol molecule than for zinc-bound water. This follows from the fact that the positive charge on NAD^+ must be assumed to induce an extensive stabilizing delocalization of the negative charge on the alcoholate ion (e.g. from the oxygen towards an α -carbon hydrogen atom in closer proximity to the nicotinamide ring of the coenzyme), while no similar stabilization of the hydroxyl ion can be obtained. Consequently, there seems to be no difficulty in accounting for the observed magnitude of the pK_a values attributed to zinc-bound water and alcohols in the enzymic complexes containing NAD^+ (Scheme 2).

Up to this point, the latter pK_a values have been assumed to reflect ionization of the isolated zinc-bound ligands. It should now be emphasized that this assumption has been made mainly to simplify the discussion. As shown by Eklund et al. [29], there is structural evidence suggesting that the actual ionizing structure exhibiting a pK_a of 7.6 in the enzyme $\cdot NAD^+$ complex may involve zinc-bound water, hydrogen bounded to a proton relay system consisting of the hydroxyl group of Ser-48 and the imidazole group of His-51. We have pointed out previously that the proton equilibrium exhibiting a pK_a of 6.4 with ethanol and benzyl alcohol may be attributable to a similar ionizing structure involving the zinc-bound alcohol [5].

Mechanism of Proton Transfer

The stoichiometry of the overall reaction catalyzed by liver alcohol dehydrogenase prescribes that one proton is transferred from the alcohol substrate to the reaction solution for every molecule of NAD^+

consumed. The idea is widely held that this proton release takes place at the binary-complex level, being associated with NAD^+ binding and the concomitant perturbation of the pK_a of zinc-bound water [1]. Examination of the transient pH changes occurring during enzyme turnover has provided evidence consistent with this idea (and consistent also with Scheme 1) [9,15].

We agree that it has been well established that NAD^+ binding results in a significant proton release from zinc-bound water at the appropriate pH. The crucial point, however, is to decide whether or not such proton release represents an obligatory step in the catalytic reaction. In this respect, the pH dependence data reported in the present investigation provide clear evidence that proton release from zinc-bound water in the binary enzyme $\cdot \text{NAD}^+$ complex represents a side reaction which is not on the main catalytic pathway. Catalytic proton transfer to or from solution occurs at the ternary-complex level (Scheme 2) and can be detected in properly designed experiments during alcohol oxidation [8], as well as during aldehyde reduction (Fig. 6).

In Scheme 2 we suggest that catalytic proton transfer is mediated by an alcohol/alcoholate ion inter-conversion of the enzyme-bound substrate, the corresponding pK_a being sufficiently low to ensure that the substrate is bound mainly in the alcoholate ion form at physiological pH. It has been argued that alcoholate ion formation on substrate binding would require too much binding energy to be catalytically advantageous from a thermodynamic point of view [22]. The main energetic problem to be solved by the enzyme, however, is to overcome the considerably higher potential barrier for hydride ion dissociation from the α -carbon of the alcohol substrate. A lowering of the latter potential barrier through alcoholate ion formation in the binding process is not only thermodynamically feasible, but appears to be the quintessence of the catalytic action of the enzyme. As indicated by our discussion in the preceding section, the binding interactions contributing to ternary-complex formation are likely to cause a sufficiently large perturbation of the pK_a of a zinc-bound alcohol to account for the experimentally observed values of pK_a in Scheme 2. Electrostatic interaction with NAD^+ can be assumed to provide a most significant contribution to this pK_a perturbation, and would be expected to induce the appearance of a partial negative charge on the α -carbon hydrogen atom to be transferred as a hydride ion. The catalytic advantage with such a mechanism for substrate binding is obvious.

It therefore seems reasonable to assume that hydride transfer to NAD^+ takes place from a negatively charged substrate molecule. This would explain the small magnitude of the substituent effect observed for the hydride transfer step, which has been previ-

ously interpreted in terms of a synchronous proton removal from the hydroxyl group of the neutral alcohol molecule [30]. Since the alcoholate ion is a thermodynamically stable species, however, there is no strong reason to believe that the hydride transfer step is truly concerted with proton transfer, at least not with proton transfer to the reaction solution. Direct evidence that ionization of the enzyme-bound alcohol may take place without concomitant hydride transfer is provided by the binding data reported for trifluoroethanol in the preceding paper. Furthermore, Schmidt et al. have shown that there is no solvent deuterium isotope effect on the hydride transfer step during aldehyde reduction, an observation which clearly indicates that hydride transfer is uncoupled from proton transfer [31].

Eklund et al. [29] proposed from crystallographic data that a proton relay system consisting of Ser-48 and His-51 may have an essential function in the catalytic reaction. The present results provide no direct information about the validity of this proposal. They do establish, however, that the catalytic function of such a system cannot be related to the mediation of proton transfer to or from zinc-bound water at the binary-complex level, but possibly to the mediation of proton transfer to or from substrate at the ternary-complex level. The proposed proton relay system might ensure a rapid neutralization and subsequent desorption of the alcoholate ion formed by hydride transfer from NADH to an aldehyde substrate. In the reverse reaction of alcohol oxidation, Ser-48/His-51 might form an essential part of the structural system which (through pK_a perturbation) facilitates alcoholate ion formation and subsequent hydride transfer to NAD^+ . Since there is no experimental evidence that a Ser-48/His-51 proton relay system is actually operative during catalysis in solution, it seems unnecessary to discuss the mechanistic implications of the proposal of Eklund et al. in further detail for the time being.

Previous conclusions that catalytic proton transfer in the liver alcohol dehydrogenase reaction is associated with coenzyme binding [1] have placed this enzyme in a unique position among the dehydrogenases as concerns the mechanism for proton transfer from substrate to solution. The present investigation seems to establish that liver alcohol dehydrogenase, by analogy to other dehydrogenases [2], operates by a reaction mechanism in which hydride transfer is directly coupled to (and facilitated by) proton transfer to or from solution at the ternary-complex level.

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Substituent Effects on the Ionization Step Regulating Desorption and Catalytic Oxidation of Alcohols Bound to Liver Alcohol Dehydrogenase

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1. Transient-state kinetic methods, based on the use of pyrazole as a displacing reagent and reporter ligand, have been applied to examine the pH dependence of rate and equilibrium constants for 2-chloroethanol and 2,2-dichloroethanol binding to the binary complex formed between liver alcohol dehydrogenase and NAD^+ .

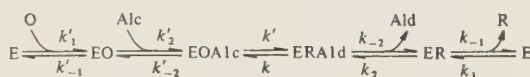
2. The apparent affinity of the enzyme $\cdot \text{NAD}^+$ complex for the examined alcohols is dependent on two proton dissociation equilibria. One of these equilibria affects the rate of alcohol association to the binary complex with a ligand-independent pK_a value of 7.6, attributable to ionization of zinc-bound water in the enzyme $\cdot \text{NAD}^+$ complex. The second proton dissociation equilibrium regulates the rate of alcohol desorption from the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex and exhibits a pK_a value of 5.4 and 4.5, respectively, with 2-chloroethanol and 2,2'-dichloroethanol as the ligand. Steady-state kinetic data are reported which indicate that the pK_a -5.4-equilibrium controls also the apparent rate of enzymic hydride transfer from 2-chloroethanol to NAD^+ .

3. These results lend strong support to the mechanism of enzyme action proposed by Kvassman and Pettersson according to which the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex participates in an obligatory proton dissociation step which regulates both desorption and catalytic oxidation of the alcohol ligand. The corresponding pK_a value is shown to be linearly dependent on the pK_a of the free alcohol ligand with a regression coefficient (Brönsted α value) of about 0.6. The latter observation provides direct evidence that the effect of pH on the reactivity of the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex reflects an alcohol/alcoholate ion equilibration of the enzyme-bound substrate.

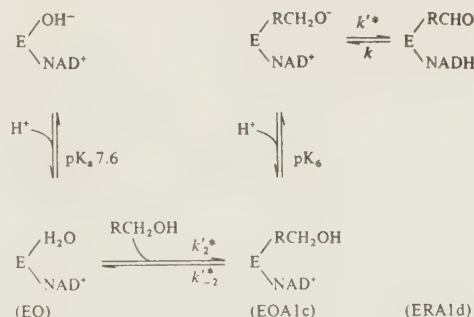
4. The particular mode of binding and properties of the active-site zinc ion in liver alcohol dehydrogenase suggest that the catalytic function of the metal ion can be related primarily to facilitation of the process of alcohol oxidation through facilitation of the alcoholate ion formation step now established to precede hydride transfer to NAD^+ .

Liver alcohol dehydrogenase catalyzes the oxidation of alcohols by NAD^+ . The enzyme operates by an effectively ordered ternary-complex mechanism (Scheme 1), coenzyme binding preceding binding of the alcohol substrate [1]. Investigations of the effect of pH on the catalytic steps of alcohol binding [2,3] and ternary-complex interconversion [4,5] have shown that the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex participates in a proton dissociation equilibrium which regulates the reactivity of this reaction intermediate. Alcohol desorption occurs exclusively from the protonated form of the ternary complex, while hydride transfer from the alcohol substrate to NAD^+ requires the unprotonated form of the complex.

It has been suggested in previous reports from our laboratory [3,5] that the effect of pH on the reactivity of the enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complex derives from an alcohol/alcoholate ion equilibration of the enzyme-bound substrate (Scheme 2). Determinations of the corresponding acid dissociation constant (K_a in Scheme 2) have failed to provide evidence in support of this suggestion, however. Estimates of pK_a close to 6.4 have been obtained with all substrates hitherto examined (ethanol [4], benzyl alcohol [3,5], and naphthyl alcohol [3]), which might seem to favour the alternative possibility that the reactivity of the ternary complex



Scheme 1. The ordered ternary-complex mechanism usually considered for evaluation of the kinetics of liver alcohol dehydrogenase catalysis. E, R, and O denote enzyme, NADH , and NAD^+ , respectively. Alc stands for an alcohol substrate, and Ald for the corresponding aldehyde product.



Scheme 2. Mechanism proposed [3] to account for the pH dependence of alcohol binding and ternary-complex interconversion in the liver alcohol dehydrogenase reaction.

Enzyme, Liver alcohol dehydrogenase or alcohol: NAD^+ oxidoreductase (EC 1.1.1.1).

is controlled by a substrate-independent ionizing group on the enzyme [1,4,6–11]. On the other hand, binding of the inhibitory substrate analogue trifluoroethanol has been shown to conform to Scheme 2 with $pK_6 = 4.3$ [2]. The latter observation might indicate that sufficiently drastic changes of the electronic properties of the alcohol ligand do result in appreciable changes of the magnitude of pK_6 .

To test the latter idea, we have carried out experiments aimed at a determination of pK_6 for two alcohols (mono-chloroethanol and dichloroethanol) with electronic properties intermediate between those of ethanol and trifluoroethanol. The results reported in the present paper provide clear evidence that there is a linear correlation between pK_6 for an enzyme · NAD⁺ · alcohol complex and the pK_a of the free alcohol ligand. This observation lends strong experimental support to the proposal in Scheme 2 that the pK_6 step reflects an alcohol/alcoholate ion equilibration of the enzyme-bound substrate.

EXPERIMENTAL PROCEDURE

Unless otherwise stated, Materials and Methods used in the present investigation were the same as detailed in the preceding paper [12]. 2-Chloroethanol and 2,2-dichloroethanol of analytical grade were obtained from Fluka AG and used without further purification.

Steady-state rate parameters (ϕ_i) for the enzymic oxidation of 2-chloroethanol (Alc) by NAD⁺ (O) were calculated from the Dalziel [13] relationship

$$\frac{v}{c_E} = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[Alc]} + \frac{\phi_{12}}{[O][Alc]} \quad (1)$$

Steady-state reaction velocities (v) with enzyme active-site concentrations (c_E) within the range 1–5 μ M were determined at 25 °C in 0.1 M ionic strength phosphate (pH 6–9) or succinate (pH 5–6) buffer by standard spectrophotometric methods [13] using a Hitachi model 100-60 spectrophotometer. Rate constants for coenzyme dissociation from the enzyme · NADH complex in 0.1 M ionic strength succinate buffer, pH 5–6, were determined by the displacement method previously used for analogous rate constant determinations at higher pH [14].

The kinetics of displacement of 2-chloroethanol and 2,2-dichloroethanol from the respective enzyme · NAD⁺ · alcohol complex were examined using pyrazole as a displacing reagent and reporter ligand. Experiments were performed and evaluated as described previously for the corresponding reaction involving trifluoroethanol as the displaced alcohol ligand [2,12]. 2-Chloroethanol forms a productive ternary complex with enzyme and NAD⁺ [9], but catalytic breakdown of this complex is slow enough ($< 0.1 \text{ s}^{-1}$) to have no significant effect on the rapid process of displacement of the alcohol by pyrazole. Pre-mixing conditions in the displacement experiments involving 2-chloroethanol had to be modified, however, such that enzyme, preincubated with NAD⁺ only, was mixed with a solution containing both pyrazole and 2-chloroethanol. With the reactant concentrations used, the alcohol equilibrates much more rapidly than pyrazole with the enzyme · NAD⁺ complex. Pre-equilibration between the binary complex and the alcohol ligand will be essentially completed within the dead-time (about 2 ms) of the stop-flow apparatus and result in ternary-complex formation to approximately the same extent as would have been obtained by

preincubation of the enzyme · NAD⁺ complex with 2-chloroethanol in the absence of pyrazole. The above modification of the pre-mixing conditions, therefore, will have a minor effect only on the amplitude of the exponential transient governing the displacement reaction and no effect at all [15] on the rate of the transient.

Displacement reactions performed at pH values below 6 were initiated by the pH-jump method described previously [2], except that pre-equilibration of the enzyme with NAD⁺ (or with NAD⁺ and 2,2-dichloroethanol) was carried out in unbuffered sodium sulphate solutions adjusted to pH 10 instead of pH 6.

RESULTS

Relationships Used for Estimation of pK_6

The data now reported for the liver alcohol dehydrogenase system will be evaluated and interpreted with the standard assumption that proton dissociation steps equilibrate rapidly in comparison to other reaction steps in the kinetic schemes considered. Scheme 2 then prescribes that two rate constants (k' and k'_{-2}) in Scheme 1 are affected by the pK_6 equilibrium [3], the pH dependence of the rate constants being given by

$$k' = \frac{k'^*}{1 + \frac{[H^+]}{K_6}} \quad (2)$$

$$k'_{-2} = \frac{k'^*_{-2}}{1 + \frac{K_6}{[H^+]}} \quad (3)$$

Further, it follows from Scheme 2 that the effect of pH on the alcohol association rate constant (k_2 in Scheme 1) can be expressed as

$$k_2 = \frac{k_2^*}{1 + \frac{K_7}{[H^+]}} \quad (4)$$

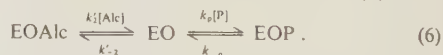
where $pK_7 = 7.6$ [3]. Eqns (3) and (4) show that the pH dependence of the equilibrium constant $K'_2 = k'_{-2}/k_2$ for alcohol dissociation from the productive ternary complex in Scheme 1 is given by

$$K'_2 = K_2^* \cdot \frac{1 + \frac{K_7}{[H^+]}}{1 + \frac{K_6}{[H^+]}} \quad (5)$$

when Scheme 2 applies.

Alcohol Dissociation Rates

Eqn (6) shows the kinetic scheme for displacement of an alcohol ligand (Alc) from the ternary complex formed with liver alcohol dehydrogenase (E) and NAD⁺ (O) by the addition of a competitive ligand such as pyrazole (P):



When the competing ligands are present in large excess to enzyme, the kinetics of the displacement reaction will be governed by two exponential transients, the fast one of which would be expected to be too rapid to be detected by stop-flow

techniques [14]. The apparent first-order rate constant (r) for the slow transient is given by

$$r = \frac{k_{-p}k'_2[\text{Alc}] + k'_{-2}k_p[\text{P}] + k_{-p}k'_{-2}}{k'_2[\text{Alc}] + k'_{-2} + k_p[\text{P}] + k_{-p}} \quad (7)$$

which may be reduced and written [14] as

$$\frac{1}{r} = \frac{1}{k'_{-2}} \left(1 + \frac{k'_2[\text{Alc}]}{k_p[\text{P}]} \right) \quad (8)$$

when

$$\frac{k_p[\text{P}]}{k_{-p}} \gg \frac{k'_2[\text{Alc}]}{k'_{-2}} \gg 1. \quad (9)$$

Methods based on Eqns (6–9) and the use of pyrazole as a displacing reagent and reporter ligand have been previously applied for examination of the pH dependence of the off-velocity constant (k'_{-2}) for trifluoroethanol binding to the enzyme $\cdot \text{NAD}^+$ complex [2], which made it possible to determine $\text{p}K_6$ for this inhibitory alcohol from Eqn (3). Entirely analogous displacement methods were used in the present investigation to obtain estimates of k'_{-2} for alcohol desorption from the enzyme $\cdot \text{NAD}^+$ 2,2-dichloroethanol complex at different pH. The typical results in Fig. 1 show the hyperbolic dependence on pyrazole concentration of the apparent first-order rate constant (r) for the displacement reaction at pH 7. As prescribed by Eqn (8), the reciprocal value of the intercept of the straight line fitted to the data in Fig. 1 was taken to provide a measure of the dichloroethanol dissociation rate constant at pH 7. The estimates of k'_{-2} thus obtained at different pH between 6 and 9 are given in Fig. 2 and can be seen to be approximately proportional to the hydrogen ion concentration over the examined pH range. Below pH 6, the displacement reaction became too rapid to be reliably characterized by stop-flow techniques. In contrast to the results obtained with trifluoroethanol [2], therefore, no estimate of the limiting magnitude of k'_{-2} at low pH could be obtained with dichloroethanol as the displaced ligand. This precluded direct determination of $\text{p}K_6$ for the latter alcohol by application of Eqn (3). The data in Fig. 2 do establish, however, that dichloroethanol desorption requires protonation of an ionizing group exhibiting a $\text{p}K_5$ value below 6 in the enzyme $\cdot \text{NAD}^+$ alcohol complex. The observed pH dependence of the 2,2-dichloroethanol dissociation rate constant is consistent with Scheme 2 and indicates that Eqn (3) applies with $k'_{-2} > 500 \text{ s}^{-1}$ and $\text{p}K_6 < 6$.

Rate constants k'_{-2} for 2-chloroethanol dissociation over the pH range 7–9 were similarly determined, except that pre-mixing conditions were modified in the displacement experiments to avoid interferences from catalytic oxidation of the alcohol. The results are included in Fig. 2. They show that the 2-chloroethanol dissociation rate increases linearly with increasing hydrogen ion concentration to become unmeasurably high below pH 7. This is consistent with the rate behaviour predicted by Eqn (3) for $k'_{-2} > 500 \text{ s}^{-1}$ and $\text{p}K_6 < 7$.

Alcohol Association Rates

According to Eqn (8), the relative magnitude of rate constants for alcohol (k'_2) and pyrazole (k_p) association to the enzyme $\cdot \text{NAD}^+$ complex can be determined from the slope of the straight line obtained by the plotting method in Fig. 1. Estimates of k'_2/k_p thus calculated for the displacement of

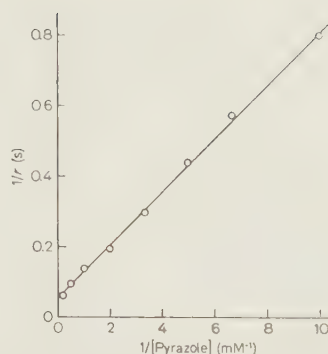


Fig. 1. Dependence on pyrazole concentration of the rate of displacement of 2,2-dichloroethanol from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Apparent first-order rate constants (r) determined from the transient absorbance changes observed at 300 nm on mixing enzyme (12 μM), preincubated with NAD^+ (1 mM) and dichloroethanol (500 μM), with varied concentrations of pyrazole at 25 $^{\circ}\text{C}$ in 0.1 M ionic strength phosphate buffer, pH 7.0

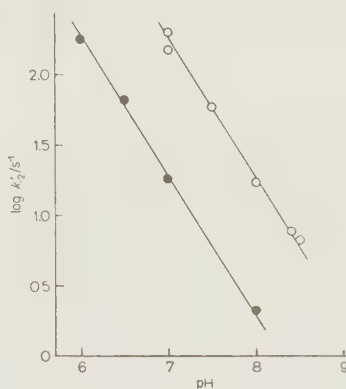


Fig. 2. Effect of pH on the rate of alcohol dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Rate constants (k'_{-2}) for 2-chloroethanol (O) and 2,2-dichloroethanol (●) dissociation determined from displacement data such as those in Fig. 1

2,2-dichloroethanol by pyrazole at different pH between 6 and 9 were found to differ insignificantly from the value (2.8) obtained for the reaction at pH 7 (Fig. 1). The quotient k'_2/k_p for 2-chloroethanol displacement by pyrazole was similarly found to remain constant (1.0) between pH 7 and 9. These observations provide evidence that k'_2 for the two alcohols exhibits the same pH dependence as k_p over the examined pH ranges. Considering the results in the preceding paper (which characterize the magnitude and pH dependence of the pyrazole association rate constant k_p), it may be concluded that the effect of pH on rate constants for 2-chloroethanol and 2,2-dichloroethanol association to the enzyme $\cdot \text{NAD}^+$ complex is adequately described by Eqn (4) with $k'_2 = 0.35$ and $1.0 \text{ s}^{-1} \mu\text{M}^{-1}$, respectively, and with $\text{p}K_7 = 7.6$

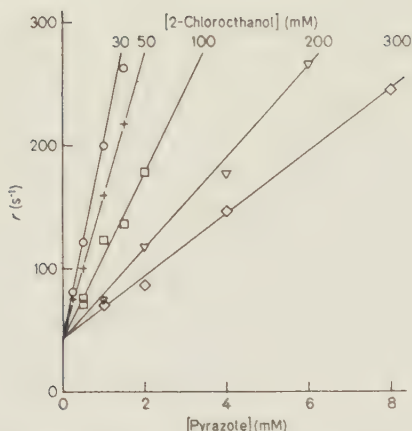


Fig. 3. Dependence on pyrazole concentration of the rate of displacement of 2-chloroethanol from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Apparent first-order rate constants (r) determined from the transient absorbance changes observed at 300 nm on mixing enzyme (20 μM), preincubated with NAD^+ (4 mM) in 33 mM sodium sulphate at pH 10, with a 0.19 M succinate buffer solution (pH 3.8) containing varied concentrations of pyrazole and 2-chloroethanol. Reactions were performed at 25°C and pH 4.0 (after mixing)

Equilibrium Constants for Alcohol Binding

The results in Fig. 2 establish that the rates of 2-chloroethanol and 2,2-dichloroethanol dissociation from the enzyme $\cdot NAD^+$ $\cdot$ alcohol complex greatly exceed the rate of pyrazole dissociation from the enzyme $\cdot NAD^+$ $\cdot$ pyrazole complex [12]. Displacement of these alcohols according to the reaction in Eqn (6), therefore, can be effected using such low concentrations of pyrazole that the relationship

$$k_p[P] + k_{-p} \ll k'_2[Alc] + k'_{-2} \quad (10)$$

holds true. Eqn (7) then reduces to

$$r = k_{-p} + \frac{k_p[P]}{1 + \frac{[Alc]}{K'_2}} \quad (11)$$

This corresponds to a kinetic situation where pyrazole binding, rather than alcohol desorption, represents the main rate-limiting step in the displacement reaction. Although no individual estimates of on-velocity and off-velocity constants for alcohol binding can be obtained under such conditions, Eqn (11) may be used for determination of the equilibrium constant K'_2 for alcohol dissociation from the ternary complex.

Apparent first-order rate constants (r) for displacement of 2-chloroethanol from the enzyme $\cdot NAD^+$ $\cdot$ alcohol complex by varied low concentrations of pyrazole were determined at different pH between 4 and 8. Measurements at pH 4 were performed for a series of fixed concentrations of the alcohol ligand. The primary plots in Fig. 3 and replot in Fig. 4 show that the observed effects of pyrazole and alcohol concentrations on r are qualitatively consistent with Eqn (11). A least-squares fit of Eqn (11) to the data in Fig. 3 gave $K'_2 = 21 (\pm 3)$ mM, $k_p = 0.39 (\pm 0.04) s^{-1} \mu M^{-1}$, and $k_{-p} = 43 (\pm 6) s^{-1}$. The latter estimates of the pyrazole asso-

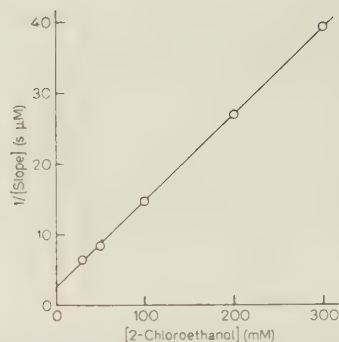


Fig. 4. Replot of slopes of the straight lines fitted to the data in Fig. 3

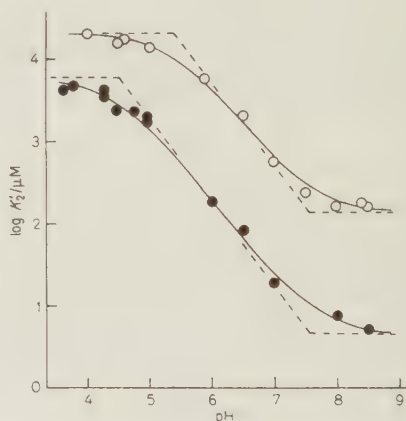


Fig. 5. Effect of pH on the equilibrium constant for alcohol dissociation from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Equilibrium constants (K'_2) for 2-chloroethanol (O) and 2,2-dichloroethanol (●) dissociation calculated from displacement data such as those in Fig. 3

ciation and dissociation rate constants at pH 4 agree well with those reported in the preceding paper, an observation which establishes also the quantitative validity of Eqn (11). Measurements of the rate of the displacement reaction at other pH values were usually carried out for a single (50–90% saturating) concentration of 2-chloroethanol. As prescribed by Eqn (11), plots of r vs pyrazole concentration were found to be linear at each fixed pH and to give intercept values agreeing with the reported [12] magnitude of the pyrazole dissociation rate constant (k_{-p}) at corresponding pH. Estimates of K'_2 , therefore, were calculated from the slope of the linear plots with the assumption that Eqn (11) applies, using the values of the pyrazole association rate constant (k_p) reported in the preceding paper.

The results thus obtained (Fig. 5) show that the 2-chloroethanol dissociation equilibrium constant, K'_2 , exhibits a pH dependence consistent with Eqn (5). Assuming that $pK_7 = 7.6$ (see preceding section), a fit of Eqn (5) to the data in Fig. 5 gave $pK_6 = 5.4 (\pm 0.2)$ and $K'_2 = 21 (\pm 4)$ mM. This estimate of K'_2 , according to the k'_2 value reported for 2-chloro-

ethanol in the preceding section, corresponds to $k'^*_2 = 7400 \text{ s}^{-1}$. Using the latter estimate of k'^*_2 , a fit of Eqn (3) to the 2-chloroethanol dissociation rate data in Fig. 2 gave $\text{p}K_6 = 5.4$. It may be concluded that the kinetic and equilibrium data now reported provide consistent evidence that 2-chloroethanol binding to the enzyme $\cdot \text{NAD}^+$ complex conforms to the mechanism in Scheme 2 with $\text{p}K_6 = 5.4$.

The validity of Eqn (11) for description of the ligand concentration dependence of the rate of displacement of 2,2-dichloroethanol by low concentrations of pyrazole was similarly established, and estimates of K'_2 for the latter alcohol were analogously determined over the pH range 4–7. The results are included in Fig. 5. They show that the pH dependence of K'_2 for 2,2-dichloroethanol conforms to Eqn (5) with $\text{p}K_7 = 7.6$, regression analysis yielding the best-fit estimates $\text{p}K_6 = 4.5 (\pm 0.2)$ and $K'_2 = 6.0 (\pm 0.8) \text{ mM}$. The latter datum, combined with the k'^*_2 value obtained for 2,2-dichloroethanol association, gives $k'^*_2 = 6000 \text{ s}^{-1}$. Using this estimate of k'^*_2 , a fit of Eqn (3) to the 2,2-dichloroethanol data in Fig. 2 gave $\text{p}K_6 = 4.5$. It may be concluded that 2,2-dichloroethanol binding to the enzyme $\cdot \text{NAD}^+$ complex, in all respects tested, conforms to the predictions of Scheme 2 with $\text{p}K_6 = 4.5$.

Enzymic Oxidation of 2-Chloroethanol

According to Eqn (2), $\text{p}K_6$ for an alcohol substrate may be determined by examination of the effect of pH on the apparent rate constant (k') for the catalytic step of hydride transfer to NAD^+ . This method has been previously applied to a number of substrates [3–5], estimates of k' being calculated from the rate of the transient burst of NADH production known to precede the steady-state phase of enzymic oxidation of typical alcohol substrates [1]. Such burst kinetics are at hand when NADH production in the hydride transfer step is more rapid than NADH desorption from the enzyme (which limits the steady-state rate of oxidation of typical alcohol substrates [1]) [5,16]. Hydride transfer from 2-chloroethanol, however, has been found to be too slow (i.e. much slower than NADH desorption) to give rise to any significant burst of NADH production and hence appears to be rate-limiting for the overall catalytic reaction with this substrate [9]. This means that the corresponding maximum steady-state turnover rate, the reciprocal value of the parameter ϕ'_0 in Eqn (1), should provide an approximate measure of the apparent rate constant k' for hydride transfer from 2-chloroethanol to NAD^+ .

Fig. 6 shows the effect of pH on the parameter ϕ'_0 for the enzymic oxidation of 2-chloroethanol by NAD^+ . The observed values of $1/\phi'_0$ are at least 20-fold lower than those reported for the NADH dissociation rate constant (k_{-1}) at corresponding pH above 6 [1,14]. Complementary determinations of k_{-1} now performed established that the same holds true also between pH 5 and 6. These observations lend credence to the previous conclusion that hydride transfer is rate-limiting for the enzymic oxidation of 2-chloroethanol [9] and indicate that the approximation $k' = 1/\phi'_0$ is valid over the pH range now examined. The data in Fig. 6, therefore, provide clear evidence that the rate constant k' for 2-chloroethanol oxidation exhibits a pH dependence consistent with Eqn (2). A fit of Eqn (2) to the data in Fig. 6 with the assumption that $k' = 1/\phi'_0$ gave $k'^* = 0.10 (\pm 0.02) \text{ s}^{-1}$ and $\text{p}K_6 = 5.5 (\pm 0.2)$. The latter estimate of $\text{p}K_6$ agrees excellently with that (5.4) obtained from the 2-chloroethanol binding data in Fig. 5. This observation lends crucial ex-

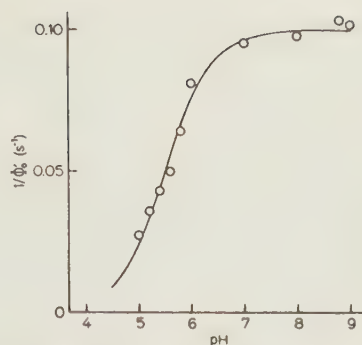


Fig. 6. Effect of pH on the maximum steady-state rate of enzymic oxidation of 2-chloroethanol by NAD^+ . Estimates of the parameter ϕ'_0 in Eqn (1) determined by standard steady-state kinetic measurements at 25°C in 0.1 M ionic strength phosphate (pH 6–9) or succinate (pH 5–6) buffer

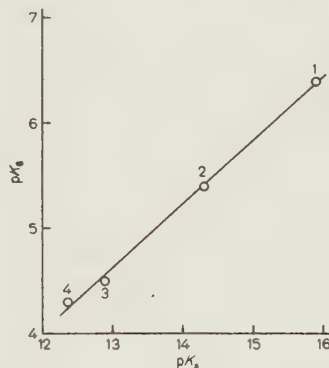


Fig. 7. Dependence of $\text{p}K_6$ for ionization of the ternary complex formed between liver alcohol dehydrogenase, NAD^+ , and alcohol ligands on the $\text{p}K_a$ of the free alcohol. Brønsted plot of $\text{p}K_6$ values reported previously [2,4] and in the present paper vs the $\text{p}K_a$ value [17] for ionization of ethanol (1), 2-chloroethanol (2), 2,2-dichloroethanol (3), and trifluoroethanol (4). The slope of the regression line drawn corresponds to a Brønsted α value of 0.61

perimental support to the proposal in Scheme 2 that ternary enzyme $\cdot \text{NAD}^+ \cdot$ alcohol complexes participate in a proton dissociation step which regulates both desorption and catalytic oxidation of the alcohol.

2,2-Dichloroethanol was not enzymatically oxidized by NAD^+ at a detectable rate between pH 5 and 9.

Substituent Effects on $\text{p}K_6$

The presence of halogen substituents at C-2 of ethanol is known to decrease the $\text{p}K_a$ for ionization of the hydroxyl group of the alcohol [17]. Considering previously reported data for ligands such as ethanol [4] and trifluoroethanol [2], the present results with 2-chloroethanol and 2,2-dichloroethanol establish that there is a similar substituent effect on $\text{p}K_6$ for ionization of the ternary complexes formed between liver alcohol dehydrogenase, NAD^+ , and alcohol ligands. This is demonstrated by the Brønsted plot in Fig. 7,

which shows that pK_a is actually linearly dependent on the pK_a [17] of the free alcohol ligand. The regression line fitted to the data in Fig. 7 corresponds to a Brönsted α value of 0.61.

DISCUSSION

Substituent Effects on Ternary-Complex Interconversion

Aldehyde formation during the catalytic action of liver alcohol dehydrogenase requires a net removal of two hydrogen atoms from the alcohol substrate. This dehydrogenation process is known to proceed at the ternary-complex level through hydride ion transfer from C-1 of the substrate to NAD^+ , combined with proton transfer from the hydroxyl group of the substrate to the reaction medium [1]. The presence of electron-withdrawing substituents in the side-chain of an alcohol substrate, therefore, can be anticipated to have a dual effect on the catalytic process of ternary-complex interconversion. The decrease in electron density at C-1 of the substrate will lower the rate of the hydride transfer step, while proton transfer will be facilitated by the decrease in electron density at the hydroxyl oxygen atom.

Kinetic studies of the enzymic oxidation of various alcohols have established that electron-withdrawing substituents significantly decrease the magnitude of the rate constant k' for ternary-complex interconversion in Scheme 1 [8,9,16]. Blackwell and Hardman [9] concluded that the comparatively small substituent effects observed are consistent with rate-limiting hydride transfer coupled with removal of the hydroxyl proton of the substrate by a suitable basic group on the enzyme, suggested to be identical with the one regulating the reactivity of the ternary enzyme $\cdot NAD^+$ $\cdot$ alcohol complex. According to this idea, the substituent effect on proton transfer appears in the same kinetic step as (and is masked by) the more pronounced substituent effect on hydride transfer, the combined effects showing up in one and the same rate constant (k^* in Scheme 2). The pK_a step in Scheme 2, then, can be envisaged to represent proton transfer from the enzymic group to solvent, a process which would be expected to exhibit no significant dependence on substrate structure. Such a reaction mechanism would be consistent with the observed constancy of pK_a for all substrates previously examined (see introduction), and the observation that trifluoroethanol binding conforms to Scheme 2 with a considerably lower pK_a value might be irrelevant. Alcohol substrates and the inhibitor trifluoroethanol could be differently bound and might then interact with entirely different ionizing groups in the ternary complexes formed.

As pointed out by Kvassman and Pettersson [3], however, the comparatively small substituent effect on ternary-complex interconversion is consistent also with the proposal in Scheme 2 that hydride transfer takes place from a negatively charged alcoholate ion. If such a reaction mechanism is at hand, the observed substituent effect on k' would represent an isolated effect on hydride transfer (deriving from the effect on k^* in Scheme 2), while the concomitant substituent effect on proton transfer should be kinetically detectable as an effect of substrate structure on the magnitude of pK_a in Scheme 2.

The present results provide clear evidence in favour of the latter alternative. They establish that binding and catalytic oxidation of the substrate now examined (2-chloroethanol) conforms to Scheme 2 with a pK_a value of 5.4, significantly lower than that (6.4) reported to regulate the enzymic oxidation of ethanol [4]. This observation demonstrates that sufficiently

pronounced alterations of the electronic properties of an alcohol substrate do result in detectable changes of the pK_a value regulating the reactivity of the productive ternary complex. The concomitant substituent effect on the actual step of hydride transfer is more drastic, as would be expected from theoretical considerations and from previously reported correlations between the rate constant k' and substrate structure [8,9]. The hydride transfer rate decreases from 130 s^{-1} with ethanol [4] to 0.1 s^{-1} with 2-chloroethanol (Fig. 6) and becomes unmeasurably low with 2,2-dichloroethanol and trifluoroethanol.

The absence of detectable enzyme activity with the latter two alcohols can thus be well understood in view of the substituent effect on the hydride transfer step, and there are no indications that the mode of binding of these inhibitory alcohols differs from that of alcohol substrates. On the contrary, it has been concluded previously that entirely analogous kinetic patterns and mechanisms apply for the binding of alcohol ligands irrespective of the actual rate of catalytic oxidation of the ligand [2,3]. The kinetic data now reported reaffirm that conclusion, which is supported also by crystallographic evidence showing that alcohol substrates and trifluoroethanol form structurally analogous ternary complexes with enzyme and NAD^+ [18]. Consequently, there seems to be no doubt that the pK_a values obtained by examination of the pH dependence of 2,2-dichloroethanol (Fig. 5) and trifluoroethanol [2] binding refer to the same ionization process as the one regulating alcohol dissociation from and catalytic turnover of the productive ternary complexes formed with 2-chloroethanol and other alcohol substrates.

It may, therefore, be concluded from the data in Fig. 7 that there is a pronounced substituent effect on the ionization step regulating the reactivity of the enzyme $\cdot NAD^+$ $\cdot$ alcohol complex, such that the corresponding pK_a value decreases systematically with an increasing number of halogen substituents (from 6.4 with ethanol to 4.3 with trifluoroethanol). This observation provides direct evidence that the substituent effect on proton transfer appears in a reaction step distinct from that of hydride transfer, which excludes that the enzymic dehydrogenation of alcohol substrates may proceed by a mechanism of truly concerted hydride and proton transfer. Recent investigations of solvent deuterium isotope effects on the catalytic reaction have provided strong evidence in the same direction [11].

Since the substituent effect on proton transfer during enzymic alcohol oxidation appears in the pK_a step in Scheme 2, this ionization step can be identified as the one representing catalytic proton transfer from substrate to solution. In other words, the proton released in the pK_a step must derive from the enzyme-bound alcohol. This means that the proton must actually be assumed to derive from the hydroxyl group of the substrate; no reasonable alternative ionizing groups are available in the alcohol ligands examined. The observation that pK_a for ionization of the enzyme $\cdot NAD^+$ $\cdot$ alcohol complex is linearly dependent on the pK_a for alcoholate ion formation from the free alcohol ligand (Fig. 7) provides crucial evidence in favour of this conclusion and the proposal in Scheme 2 that the pK_a step reflects an alcohol/alcoholate ion equilibration of the enzyme-bound substrate.

It should be emphasized at this point that proton transfer from the enzyme-bound alcohol to solution in the pK_a step may certainly be mediated by polar groups on the enzyme, e.g. by the Ser-48/His-51 proton relay system discussed by Brändén et al. [1]. If such mediating groups are strongly coupled through hydrogen bonds to the alcohol ligand, it

might be relevant in some contexts to attribute the observed pK_a values to the entire hydrogen-bonded structure, as suggested previously [5,10]. From a mechanistic point of view, however, protonation/deprotonation of the enzyme-bound substrate represents the interesting and catalytically important event in the pK_a step. Since proton transfer occurs at rates greatly exceeding those observed for other reaction steps in the enzymic reaction mechanism, it will be of minor kinetic interest to decide whether protonation/deprotonation of the substrate takes place through direct or mediated proton transfer from/to solution. Furthermore, it has been well established that alcohols are directly ligated to the active-site zinc ion in the ternary complexes formed with enzyme and NAD^+ [1,18]. This means that the negative charge generated by proton dissociation from a hydrogen-bonded system involving the alcohol ligand can be anticipated to be located mainly at the metal-bound alcohol. For these reasons, and in view of the established correlation between pK_a and the pK_a of the free alcohol (Fig. 7), it seems justified and most appropriate to attribute pK_a to ionization of the enzyme-bound alcohol whether or not proton transfer to solution is actually mediated by polar groups on the enzyme.

It would not seem appropriate, however, to attribute pK_a to ionization of a proton-transfer-mediating group itself [1,4,6–11]. The observed correlation between pK_a and the pK_a of the free alcohol ligand corresponds to a Brønsted α value of about 0.6, and hence appears to be far too pronounced to reflect secondary effects on the pK_a of an ionizing group electronically linked to the alcohol through hydrogen bonds or through common coordination to the active-site zinc ion. In particular, recent proposals must be rejected that pK_a may reflect ionization of zinc-bound water with a pK_a value decreased by penta-coordination of the alcohol to the active-site zinc ion [9–11]. Besides being difficult to reconcile with the Brønsted correlation established by the data in Fig. 7, the latter proposals seem to be qualitatively inconsistent with the observed magnitudes of pK_a (≤ 6.4). Zinc-bound water exhibits a pK_a value of 7.6 in the binary enzyme $\cdot NAD^+$ complex [1,13,19], where the metal coordinates four ligands [1]. Penta-coordination of an alcohol to zinc in this binary complex can be anticipated to reduce the coulombic force field of the metal ion, and would thus be expected to increase the pK_a of zinc-bound water [20] instead of decreasing it.

The present investigation seems to establish the kinetic validity of the reaction mechanism in Scheme 2. This kinetic scheme, as well as the observed magnitude of the Brønsted α value for the pK_a step, can be best understood in view of the previous conclusion that alcohol binding most likely proceeds by a substitution mechanism with displacement of zinc-bound water [21]. Consequently, it seems most reasonable to attribute pK_a to ionization of an alcohol molecule ligated to a tetra-coordinate active-site zinc ion.

The Catalytic Function of the Active-Site Zinc Ion

The active-site zinc ion in liver alcohol dehydrogenase has the basic function of forming an essential part of the binding site for alcohol and aldehyde substrates [1]. After ligation of its hydroxyl or carbonyl oxygen atom to the zinc ion, the substrate molecule becomes appropriately positioned in the productive ternary complex for direct hydride transfer to or from the enzyme-bound coenzyme. It seems obvious, however, that such positioning of the substrate would not necessarily require the presence of a metal ion at the binding site. This

suggests that the active-site zinc ion has an additional mechanistic function, more directly related to catalysis of the process of alcohol/aldehyde interconversion.

The involvement of the active-site zinc ion as a Lewis acid catalyst of ternary-complex interconversion has been suggested by several authors [6, 22–30]. Most proposals in that direction have focused on the process of aldehyde reduction and emphasized the polarizing effect of the zinc ion on the metal-bound substrate. Non-enzymic hydride transfer to carbonyl compounds usually requires participation of a strong Lewis acid which polarizes the reactant by binding to its carbonyl oxygen atom [24]. Attention has been drawn to the fact that enzymic hydride transfer from NADH could be similarly facilitated by electron withdrawal from the carbonyl carbon of zinc-bound aldehyde substrates [22–29].

It should be noted, however, that dehydrogenases usually participate in metabolism as bidirectional catalysts, i.e. liver alcohol dehydrogenase is likely to have a physiological function which requires effective catalysis of both aldehyde reduction and alcohol oxidation. With $NADH/NAD^+$ as the coenzymes, the thermodynamic equilibrium for alcohol/aldehyde interconversion is greatly in favour of aldehyde reduction at physiological pH [31]. This means that liver alcohol dehydrogenase would be expected to be designed primarily to facilitate the thermodynamically unfavourable process of alcohol oxidation. In our opinion, therefore, the catalytic function of the active-site zinc ion is not likely to be related primarily to ligand polarization resulting in electron withdrawal from C-1 of the metal-bound substrate. Such polarization of the substrate molecule would certainly facilitate aldehyde reduction by NADH, but the more critical process of alcohol oxidation by NAD^+ would be catalytically disfavoured to the same extent.

More direct evidence that the former (positive) effect of substrate polarization is of minor physiological significance is provided by the actual chemical reactivity of the active-site zinc ion. If Lewis acid polarization of metal-bound aldehyde substrates were of primary catalytic importance, then the substrate-binding zinc ion would be expected to exhibit strongly polarizing properties. Actually, however, liver alcohol dehydrogenase appears to be exceptional among the zinc-containing enzymes in possessing an active-site zinc ion with extremely weak Lewis acid reactivity. This is obvious both from the exceptionally weak anion-binding capacity of the zinc ion [32] and from the extraordinarily high pK_a value for ionization of zinc-bound water in the enzyme $\cdot NADH$ complex [21]. As has been pointed out previously, the exceptional properties of liver alcohol dehydrogenase in the latter respects reflect the unique mode of binding of the active-site zinc ion in this enzyme [21,32]; no less than two negatively charged amino acid residues (Cys-46 and Cys-174) are utilized for attachment of the zinc ion to the protein [1]. It can be inferred from this structural arrangement that the enzyme is designed to provide a substrate-binding metal center with drastically reduced polarizing properties.

The above discussion indicates that an understanding of this particular design of the catalytic metal center in liver alcohol dehydrogenase requires consideration primarily of the effect of the zinc ion on the catalytic process of alcohol oxidation. The present investigation seems to establish that the enzymic oxidation of alcohols proceeds by a mechanism (Scheme 2) in which proton dissociation from the enzyme-bound substrate represents an obligatory reaction step preceding hydride transfer to NAD^+ . This proton dis-

sociation step serves the obvious catalytic purpose of facilitating hydride transfer by increasing the electron density at the hydrogen atom to be transferred as a hydride ion, an effect which is directly opposed by metal-induced polarization of the zinc-bound substrate. Metal coordination of the alcohol, however, will have the additional effect of decreasing the nucleophilic reactivity of the oxygen ligand atom, i.e. the acidity of the zinc-bound alcohol will be drastically increased. Ligation of the substrate to a zinc ion with appropriate Lewis acid strength, therefore, might be catalytically advantageous with respect to the critical process of alcohol oxidation. The negative effect of metal-induced ligand polarization could be overcome by metal-induced ionization of the enzyme-bound substrate.

According to this idea, the essential catalytic function of the active-site zinc ion can be envisaged to be that of facilitating hydride transfer to NAD^+ by decreasing the pK_a for ionization of alcohol substrates in the productive ternary complex to such an extent ($pK_6 < 7$) that the metal-bound substrate is mainly in the alcoholate ion form at physiological pH. The present and previously reported results [2–5] establish that this can be accomplished with the actual design of the catalytic metal center (a tetra-coordinate zinc ion bound by one neutral and two negatively charged protein ligands). Judging from reported inner-sphere ligand field effects on the pK_a of zinc-bound water [21], substitution of the neutral protein ligand by a third negatively charged amino acid residue would be expected to render the substrate-binding zinc ion too weakly polarizing to perturb the pK_a of typical alcohol substrates (e.g. ethanol and benzyl alcohol) to values below 8 in the productive ternary complex. The utilization of less than two negatively charged protein ligands for binding of the zinc ion would certainly create a metal center capable of perturbing the pK_a of alcohol ligands to values much lower than 7, but this would not offer any significant additional physiological advantage with respect to the enzymic process of alcohol oxidation; it suffices to bring pK_6 for alcoholate ion formation to a value slightly below 7. Neither would the process of aldehyde reduction be significantly favoured by ligation of the substrate to such a more strongly polarizing metal center. The actual rate of enzymic hydride transfer from NADH to aldehyde substrates appears to be satisfactorily high (far from being rate-limiting) as it is [1, 5].

Several circumstances, in fact, would seem to render changes of the metal center towards a more polarizing design (less than two negatively charged protein ligands) catalytically unfavourable. Firstly, the negative effect on hydride transfer to NAD^+ of direct polarization of alcohol(ate) substrates would be enhanced. Secondly, substrate and water exchange at the zinc ion would be slowed down due to truly decreased rates of ligand dissociation and, in the case of ionizing ligands, due also to apparently decreased dissociation rates reflecting the additional decrease in pK_a of the ligand; the apparent rates of water and alcohol dissociation will respond strongly to pK_a perturbations because there is no significant desorption from the metal of the ionized form of the ligands [2, 3, 21]. This could result in unsatisfactorily low rates of product dissociation in reactions involving tightly bound (e.g. aromatic) substrates, at least in the process of aldehyde reduction where alcohol desorption frequently has been found to be partially rate-limiting [1]. Moreover, apparent on-velocity constants for both aldehyde and alcohol binding would decrease as a result of the decreased apparent rate of water desorption [3, 21]. It may be noted in this context that the actual structural arrangement of liver alcohol dehydro-

genase is such that zinc-bound water exhibits a pK_a of 7.6 in the enzyme $\cdot \text{NAD}^+$ complex [13, 19]. At physiological pH, therefore, zinc-bound water will be predominantly unionized even in the latter complex, which ensures rapid formation of the productive ternary complex during the enzymic oxidation of alcohols.

Finally, attention should be drawn to the fact that the structural arrangement of the catalytic metal center may also affect the process of coenzyme binding. In particular, NAD^+ binding would be expected to be strongly dependent on the effective charge on the active-site zinc ion. The oxidized form of the coenzyme is not particularly firmly bound at physiological pH [1], and an additional decrease in affinity of the enzyme for NAD^+ (due to stronger electrostatic interaction with the zinc ion) would seem to be clearly disadvantageous with respect to the critical process of alcohol oxidation. This could be an important factor favouring the actual structural arrangement where the active-site zinc ion is bound by two negatively charged protein ligands.

Summing up the above discussion, it appears that the catalytic function of the active-site zinc ion in liver alcohol dehydrogenase should not be related primarily to direct polarization of the metal-bound substrate. On the contrary, the catalytic metal center in this zinc-containing enzyme appears to be uniquely designed to avoid the mechanistic drawbacks of substrate binding to a strongly polarizing metal ion. The particular mode of binding of the active-site zinc ion in liver alcohol dehydrogenase can be well understood with the assumption that the metal ion functions primarily as a Lewis acid catalyst of alcohol oxidation [6, 30] by causing an appropriate ($pK_6 < 7$) perturbation of the pK_a for ionization of the metal-bound substrate. As has been previously discussed in detail [3], factors other than metal coordination (mainly electrostatic interaction with NAD^+) contribute most significantly to the net pK_a perturbation for zinc-bound alcohols in the enzyme $\cdot \text{NAD}^+$ alcohol complex. This explains why a zinc ion with drastically reduced polarizing properties is sufficient to fulfil the catalytic function of ensuring that the substrate is predominantly in the alcoholate ion form in the productive ternary complex at physiological pH.

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